IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicant: | Sauvageau et al. | | |
| Docket No.: | FC 14518-24 | Confirmation No. | 7319 |
| Serial No.: | 10/727,580 | Group Art: | 1636 |
| Filing Date: | December 05, 2003 | Examiner: | Dunston, Jennifer Ann |
| Title: | STEM CELL EXPANSION ENHANCING FACTOR AND METHOD OF USE | | |

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**DECLARATION UNDER 37 CFR 1.132**

Dear Sir:

1. I, Denis-Claude Roy, M.D., Ph.D., F.R.C.P., am the Director of the Research Centre of the Hospital Maisonneuve-Rosemont in Montreal, Canada and the Director of the Cellular Therapy Research Laboratory of the Hospital.

2. My main research topics for the last 20 years have been the immunobiology of human leukemia and lymphoma stem cells, allogeneic and autologous stem cell transplantation, and engineering of hematopoietic cell grafts including immunotherapy and stem cell expansion. I have published more than 50 articles on these topics. Please find enclosed a copy of my resume.

Long Felt Need

3. Patients in need for hematopoietic stem cells (HSCs) transplantation include patients with blood cancer including leukemia, lymphoma or myeloma. Patients with certain forms of solid cancers may also benefit from HSCs transplantation including those having testicular andrenal cancer, and some non-malignant disorders, such as aplastic anemia and inherited disorders, such as thalassemia and sickle cell anemia.

4. According to the World Health Organization, more than 4 million people were diagnosed with cancer in Western Europe and North America. The number of patients having blood cancer was about 265,000 in 2007. Half of these patients die annually, the majority of them could have been considered for an HSC transplantation.

5. Typically, about 25% of patients in need for HSCs transplantation find a donor amongst HLA matched siblings. Another 25% find a HLA matched unrelated donor. The remaining 50% do not find an HLA matched donor, are generally not grafted and die.

6. Current options outside of HLA matched grafts (related or unrelated) are transplantation of umbilical cord blood isolated stem cells or transplantation of mismatched grafts (grafts from HLA mismatched donors). The first option has very limited use because the number of HSCs in one cord is insufficient for one transplantation: it is believed that blood from about two-three cords would generally be required for most patients. Combining the blood from more than one umbilical cord is an experimental procedure that is costly and that raises a number of biological concerns. The second option has limited applicability because it necessitates the collection of very high numbers of stem cells. Not all donors mobilize sufficient numbers of HSCs in their peripheral blood to proceed to such a transplant. Even when it is possible to obtain sufficient

numbers of HSCs, several collections are necessary and it would be extremely useful to identify a strategy to expand the number of HSCs ex vivo, in order to facilitate such transplants.

7. A method for expanding HSCs would save the lives of many patients who currently do not have access to HLA matched grafts. It would also facilitate sample collection for transplantation in HLA matched grafts.

8. Providing grafts for patients without HLA matched donors: A method for expanding HSCs could generate a sufficient amount of HSCs for transplantation from a single umbilical cord blood sample. Similarly, he method could be applied on HLA-mismatched grafts to enrich the samples in HSCs. Such a method would therefore significantly increase the number of patients who have access to HSC transplantation and has the potential of saving the lives of many of these patients.

9. Facilitating sample collection from HLA matched donors. Samples used for transplantation can be obtained from two different sources: 1) Bone marrow samples: such samples can be obtained with a needle biopsy typically on the back of the pelvis. Because this requires surgery, it is not the preferred method; 2) Mobilized peripheral blood cells (MPBC): After the donor has received a drug inducing bone marrow cells to migrate into blood, their blood sample is obtained and stem cells are recovered from the sample. The procedure is repeated until a sufficient amount of progenitor cells is obtained.

10. A method of expanding HMCs could thus be applied on the MPBCs samples and reduce the number of sample collections or the amount cells needed to obtain a sufficient amount of stem cells.

11. A method of expanding HSCs could also have other applications. It could be used to expand HSCs in manipulated grafts for instance. Bone marrow and peripheral blood cells samples can be collected from cancer patients

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and reintroduced into them after having been treated with chemotherapy or antibodies and magnetic beads, for example, to remove tumor cells present in the sample. As mentioned above, chemotherapy and other cell manipulation strategies also lead to the loss of stem cells. Hence a method of expanding HSCs could advantageously be applied to the sample after tumor removal.

12. HSCs have also been used to repair damaged tissues such as heart, neurologic tissue, cartilage, etc. For example, HSC have been injected either into coronaries or heart muscle of patients that had suffered a heart attack. The injection of such cells has lead to improved heart function. The problem with these approaches is to obtain sufficient numbers of HSCs. Currently, HSCs are obtained from the bone marrow cells after repeated punctures into the pelvic bone. The present strategy of HSC expansion would enable to limit the number of bone marrow punctures, thus obviating the need for extensive anesthesia. Alternatively, HSCs could be obtained from the peripheral blood, where they are circulating in low numbers, and expanded *ex vivo*, a procedure that could eliminate the need for bone marrow puncture. The expanded HSCs could then be used to repair damaged tissues.

13. Finally, HSCs also have the ability to improve engraftment of solid organs (kidney, and potentially heart, liver and pancreas). Expansion of HSCs from solid organ donors and the injection of these cells concomitantly with the transplanted organ has the potential to facilitate acceptance of the organ by the patient.

14. Despite the urgent need for a method of expanding HSCs, there is still currently no approved method for a safe and efficient expansion of HSCs for use in stem cells transplantation.

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15. I have been working with HOXB4 since at least as early as 2003. The first technique using HOXB4 tested to induce HSC expansion was a transfection of HSCs with a HOXB4 cDNA construct.

16. This method however possesses significant drawbacks that could hamper its clinical use.

17. Insertional mutagenesis in particular has been a long-term concern specifically in the context of gene therapy and HSC manipulation. It is a mutagenesis of DNA by the insertion of one or more bases in the genome of the host bone marrow cell. It can lead to leukemia.

18. The leukemogenic potential of HOXs delivered stably by gene transfer has been documented. In particular, evidence of leukemogenic potential in large animal models such as dog and monkey has been most recently evidenced.

19. The following references describe the effects of insertional mutagenesis generally observed in the context of transfection or transformation of HSCs with constructs such as HOXB4; 1) Baum, C. (2007). Insertional mutagenesis in gene therapy and stem cell biology. Curr. Opin. Hematol. *14*, 337-342; 2) Baum, C., Dullmann, J., Li, Z., Fehse, B., Meyer, J., Williams, D.A., and von Kalle, C. (2003). Side effects of retroviral gene transfer into hematopoietic stem cells. Blood *101*, 2099-2114; 3) Pineault, N., Abramovich, C., Ohta, H., and Humphries, R.K. (2004). Differential and common leukemogenic potentials of multiple NUP98-Hox fusion proteins alone or with Meis1. Mol. Cell. Biol. *24*, 1907-1917; and 4) Zhang, X., Beard, B.C., Trobridge, G.D., Wood, B.L., Sale, G.E., Sud, R., Humphries, R.K., and Kiem, H. J. Clin. Invest. 118, 1502-1510. High incidence of leukemia after stem-cell gene therapy in large animals with a HOXB4-expressing vector. Journal of Clinical Investigation. Please find enclosed copies of the full text or of the abstract of these references.

20. There is thus an urgent need for an efficient non-gene delivery method of expanding HSCs avoiding the drawbacks of HOXB4 retroviral gene transfer and enabling safe and efficient bone marrow transplantations in patients in need thereof.

Commercial success

21. On September 2005, I and other colleagues of Hôpital Maisonneuve-Rosemont have obtained a pre-IND visit to request approval of health Canada to conduct clinical studies with the HOXB4 construct of the present invention. Health Canada's response was very positive and work is underway to satisfy the Government requirements.

22. To my knowledge, there is no other clinical trial currently ongoing for methods of expanding HSCs for bone marrow transplantation that demonstrated efficacy in HSC expansion.

23. I further declare that all statements made herein are of my own knowledge and are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

2008/4/17.
Date

Denis Claude Roy
Denis-Claude Roy

# CURRICULUM VITAE

**NAME:** DENIS CLAUDE ROY

**ADDRESS:** 5276, Avenue des Tillleuls, Montréal, Québec H1T 2H6

**DATE OF BIRTH:** February 1, 1958

**PLACE OF BIRTH:** Longueuil, Canada

## EDUCATION:

| | |
|---|---|
| 1977 | D.E.C. Collège André-Grasset |
| 1982 | M.D. Université de Montréal |

## POSTDOCTORAL TRAINING:

**Internship and Residencies:**

| | |
|---|---|
| 1982-1983 | Intern in Internal Medicine, Saint-Luc Hospital, Montreal, Canada |
| 1983-1985 | Resident in Internal Medicine, Saint-Luc and Sacré-Coeur Hospitals, Montreal, Canada |

**Fellowships:**

| | |
|---|---|
| 1985-1987 | Clinical Fellow in Hematology, Maisonneuve-Rosemont, Notre-Dame, Sainte-Justine and Hotel-Dieu Hospitals, Montreal, Canada |
| 1987-1990 | Research Fellow in Medicine, under the direction of Dr. Jerome Ritz, Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, MA, U.S.A. |

## LICENSURE AND CERTIFICATION:

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|---|---|
| 1983 | Quebec License Registration, No. 83249 |
| 1983 | Licensure of the Medical Council of Canada, No. 55458 |

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|---|---|
| 1985 | National Board of Medical Examiners (U.S.A.), Certificate No. 272344 |
| 1985 | American Board of Internal Medicine, Certificate No. 101596 |
| 1986 | Quebec Board of Internal Medicine, Certificate No. 10672 |
| 1986 | Royal College of Physicians and Surgeons of Canada, Specialty: Internal Medicine, Certificate No. 342161 |
| 1987 | Quebec Certificate of Specialist in Hematology, Certificate No. 11185 |
| 1987 | Royal College of Physicians and Surgeons of Canada, Subspecialty: Hematology, Certificate No. 342161 |
| 1987 | Massachusetts License, Registration No. 58705 |
| 1988 | American Board of Internal Medicine, Subspecialty: Hematology, Certificate No. 101596 |

**ACADEMIC APPOINTMENTS**:

| | |
|---|---|
| 1987-1990 | Research Associate in Medicine, Harvard University, Boston, MA |
| 1990-1996 | Assistant Professor, Department of Medicine, University of Montreal, Montreal, Quebec |
| 1996-2004 | Associate Professor, Department of Medicine, University of Montreal, Montreal, Quebec |
| 2002-present | Adjunct Professor, Department of Experimental Medicine, McGill University, Montreal, Quebec |
| 2004-present | Professor of Medicine, Department of Medicine, University of Montreal, Montreal, Quebec |

**HOSPITAL APPOINTMENTS**:

| | |
|---|---|
| 1987-1990 | Research Associate, Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, MA |

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| 1987-1990 | Clinical/Research Fellow, Department of Medicine, Brigham and Women's Hospital, Boston, MA |
| 1990-1999 | Active Member, Department of Hematology, Maisonneuve-Rosemont Hospital, Montreal, Quebec. |
| 1990-2000 | Consultant Member, Department of Medical Biology and Departement of Medicine, Montreal Heart Institute, Montreal, Quebec. |
| 1990-present | Active Member, Departement of Medicine, Maisonneuve- Rosemont Hospital,<br>Montreal, Quebec. |
| 1999- present | Active Member, Department of Laboratories,<br>Maisonneuve-Rosemont Hospital, Montreal, Quebec. |
| 1999- present | Active Member, Oncology Program,<br>Maisonneuve-Rosemont Hospital, Montreal, Quebec. |
| 2000- present | Associate Member, Department of Medical Biology and Departement of Medicine, Montreal Heart Institute, Montreal, Quebec. |

**OTHER PROFESSIONAL POSITIONS**:

| | |
|---|---|
| 1990-present | Director, Hematopoietic Cell Therapy Clinical Laboratory and Program, Maisonneuve-Rosemont Hospital |
| 1990-present | Director, Cellular Therapy Research Laboratory, Maisonneuve-Rosemont Hospital |
| 1991-1997 | Examination Board, Substitute, Quebec College of Physicians, Section: Hematology |
| 1997-1999 | Examination Board Member, Royal College of Physicians and Surgeons of Canada, Section: Hematology |
| 1998-2004 | Inspector, Foundation for the Accreditation of Cell Therapy (FACT) |
| 2006-present | Director, Research Centre, Hopital Maisonneuve-Rosemont |

**AWARDS AND HONORS**:

| | |
|---|---|
| 1975 | Silver Medal from Governor General of Canada |
| 1976 | Academic Achievement Award, College André-Grasset |

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| 1987 | Dean's Award, University of Montreal Medical School |
| 1987 | Lederle Research Award |
| 1993 | First Prize, Clinical Research, Annual Research Meeting, Maisonneuve-Rosemont Hospital |

**RESEARCH AWARDS:**

| | |
|---|---|
| 1978 | Studentship, Ministry of Health and Welfare of Quebec, Canada |
| 1979 | Studentship, Medical Research Council of Canada |
| 1987-1990 | Fellowship, Medical Research Council of Canada |
| 1990-1994 | Scholarship, Junior I, Fonds de la recherche en santé du Québec |
| 1994-1998 | Scholarship, Junior II, Fonds de la recherche en santé du Québec |
| 1998-2002 | Scholarship, Senior, Fonds de la recherche en santé du Québec |

**COMMITTEE ASSIGNMENTS**

**International and National**

| | |
|---|---|
| 1994 | Member, Program Committee, Fourth International Marrow Transplant Symposium. |
| 1995 | Advisory Committee, Pfizer Canada: Antifungal prophylaxis for bone marrow transplant patients, Seattle, Washington. |
| 1995-2002 | Vice-Chairman, Local Organizing Committee for the Year 2002 Annual Meeting of the International Society for Experimental Hematology (ISEH) |
| 1997-1999 | Member, Organizing Committee, Frontiers of Malignant Hematology, Second International Conference – 1999. |
| 1998 | Advisory Committee, National Forum on Hematopoietic Stem Cell Transplantation from Unrelated Donors. Ottawa, Canada. |
| 1998-2000 | Member, Searle Oncology Canadian Advisory Panel. |

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| 1998-present | Executive Committee – Hematology Disease Site, Clinical Trials Group, National Cancer Institute of Canada. |
| 1999 | Canadian Non-Hodgkin's Lymphoma Advisory Panel, Schering Canada, VII International Conference of Malignant Lymphoma, Lugano, Switzerland. |
| 2000 | Photodynamic Therapy Advisory Committee, Theratechnologies Inc., Montreal, Canada. |
| 2000-2002 | Organizing Committee, Eight Biennial Canadian Bone Marrow Transplant Group Meeting 2002, Halifax, N.S. |
| 2001 | Canadian Lymphoma Workshop, National Cancer Institute of Canada (NCIC) – Amgen, Montreal, Canada. |
| 2001-2005 | Member, Quebec Provincial Council to Fight Cancer – Practice Evolution Committee, Hematological cancers. |
| 2002-2004 | Director at-large, Executive Board, Canadian Blood and Marrow Transplant Group. |
| 2002-present | Acute Leukemia/Stem Cell Transplantation Working Group, National Cancer Institute of Canada (NCIC). |
| 2003 | Co-Chairman, Organizing Committee, Lymphoma Symposium, Mount Stephen Club, Sept. 26, 2003, Montreal, Canada. |

**University**

| | |
|---|---|
| 1983-1984 | Internal Committee, Association of Residents and Interns, Université de Montréal, Canada |
| 1992-1998 | Member, Hematologic-Oncology Study Section, Réseau inter-hospitalier de cancérologie de l'Université de Montréal (RICUM) |
| 1994-present | Committee for Hematology Training Program, Université de Montréal |
| 1997 | Committee for Selection of Head of the Department of Microbiology, University of Montreal Hospital Center (CHUM). |
| 2002-present | Promotion Committee for the Department of Medicine, Université de Montréal. |

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| 2004-2005 | Committee for Selection of Head of the Department of Microbiology-Immunology, University of Montreal. |

**Hospital**

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|---|---|
| 1983-1984 | Education Advisory Committee, Saint-Luc Hospital |
| 1983-1984 | Executive Committee, Saint-Luc Hospital Interns and Residents Association |
| 1990-2000 | Multidisciplinary Oncology Committee Maisonneuve-Rosemont Hospital |
| 1991-1996 | Clinical Research Scientific Review Committee, Maisonneuve-Rosemont Hospital |
| 1992-1993 | Committee for Selection of Head of the Department of Ophtalmology, Maisonneuve-Rosemont Hospital |
| 1992-1993 | Committee for Graduate Studies, Maisonneuve-Rosemont Research Center |
| 1993 | Scientific Advisory Committee, Institut de médecine de la reproduction de Montréal |
| 1993 | Committee for Selection of Head of the Department of Pediatrics, Maisonneuve-Rosemont Hospital |
| 1994-1995 | Committee for Selection of Head of the Department of Surgery, Maisonneuve-Rosemont Hospital. |
| 1997 | Committee for Selection of Head of the Department of Ophtalmology, Maisonneuve-Rosemont Hospital. |
| 1998 | Committee for Selection of Head of the Department of Anatomo-Pathology, Maisonneuve-Rosemont Hospital. |
| 1999-present | Committee for Selection of Head of the Department of Radiation-Oncology, Maisonneuve-Rosemont Hospital. |
| 2002 | Human Ethics Committee, Hôpital Maisonneuve-Rosemont. |
| 2002-2003 | Committee for Selection of Head of the Department of Microbiology, Hôpital Maisonneuve-Rosemont. |

**Scientific Review Panels**

| | |
|---|---|
| 1990-1993 | Member, Scientific Review Committee, Cancer Research Society |
| 1991-1993 | Member, Scientific Review Committee, Clinician-Scientist Status, Fonds de la Recherche en Santé du Québec |
| 1993-1995 | Member, Scientific Review Committee, Clinician-Scientist and Career Research Scientist Awards, Fonds de la Recherche en Santé du Québec. |
| 1995-1997 | Vice-Chairman, Scientific Review Committee, Clinician Scientist and Career Research Scientist Awards, Fonds de la Recherche en Santé du Québec. |
| 1995-1996 | Member, Scientific Review Committee, Cancer Research Society |
| 1997-1998 | Chairman, Scientific Review Committee, Clinician Scientist Awards, Fonds de la recherche en santé du Québec. |
| 1998-1999 | Member, Scientific Review Committee, Cancer Research Society |
| 2000 | Member, Canadian Scientific Advisory Committee, 24th World Congress of the International Society of Hematology (2000). |
| 2000 | Member, Scientific Review Panel: Cell Processing – Purging and Depletion, 2000 Annual Meeting of the American Society of Hematology (ASH). |
| 2002 | Member, Scientific Review Panel: Leukemia - Clinical, 2002 Annual Meeting of the International Society for Experimental Hematology (ISEH). |
| 2003 | Member, Scientific Review Committee, AIDS and Infectious Diseases Network, Fonds de Recherche en Sante du Quebec. |
| 2003-present | Scientific Review Committee, Leukemia Cell Bank of Quebec. |
| 2004 | Member, Scientific Review Committee, René Malo Initiative Project : Montreal Cancer Institute, Canada. |
| 2005 | Member, Scientific Review Committee, Leukemia Lymphoma Society of Canada |

**Scientific Evaluation (Journals) :**

Blood
Vox Sanguinis
Bone Marrow Transplantation
Journal of Clinical Oncology
Journal of Immunological Methods
American Journal of Reproductive Immunology
Clinical Cancer Research
Journal of Palliative Care
The Hematology Journal

**MEMBERSHIPS IN PROFESSIONAL SOCIETIES:**

| | |
|---|---|
| 1980- 2005 | Association des médecins de langue française du Canada (AMLFC) |
| 1986- present | Royal College of Physicians, Fellow in Medicine (FRCPC) |
| 1991- present | American Society of Hematology (ASH) |
| 1991- present | Canadian Bone Marrow Transplantation Group (CBMTG) |
| 1993- 2005 | American Association for the Advancement of Science (AAAS) |
| 1995- present | American Society for Blood and Marrow Transplantation (ASBMT) |
| 1996-2001 | International Society for Hematotherapy and Graft Engineering (ISHAGE) |
| 2001-present | International Society for Cell Therapy (ISCT) |
| 2002-present | International Society for Experimental Hematology (ISEH) |

**MAJOR RESEARCH INTERESTS:**

1. Immunobiology of human leukemia and lymphoma stem cells.
2. Allogeneic and autologous stem cell transplantation.
3. Engineering of hematopoietic cell graft: Selective elimination of alloreactive T cells and malignant cells, immunotherapy and stem cell expansion.
4. Novel therapeutic agents.

**TEACHING EXPERIENCE:**

| | |
|---|---|
| 1990-1993 | Director, Scientific activities of the Dept. of Hematology, Maisonneuve-Rosemont Hospital |
| 1990-1993 | Lecturer, Hematology Course: MMD-2207, Faculty of Medicine, University of Montreal |
| 1991-1996 | Lecturer, Workshops for Hematology Residents, University of Montreal |
| 1991-1997 | Lecturer, Post-Graduate Course: Medical Immunology MCB-6034, Dept. of Microbiology and Immunology, University of Montreal |
| 1991-2005 | Lecturer, Transplantation Immunology Seminar, Surgery Training Program, University of Montreal |
| 1992-1998 | Lecturer, Department of Internal Medicine: Leukemias and Myelodysplasias, Maisonneuve-Rosemont Hospital |
| 1993 | Lecturer, Post-Graduate Course: New Developments in Molecular Biology, BIM-6030, Department of Molecular Biology, University of Montreal |
| 1994-2000 | Lecturer, Post-Graduate Course: Immunopharmacology: PHL-6093, University of Montreal |
| 1996-2000 | Lecturer, Postgraduate Hematology Programs Lecture Series, University of Montreal and McGill University. |
| 2000-present | Lecturer, Postgraduate Hematology Programs Lecture Series, University of Montreal. |
| 2002-present | Chairman of the Course (45 hours – 3 credits) and Lecturer, Biomedical Sciences - Fundamental and Clinical Hematology: SBM-3003, University of Montreal. |
| 2003-present | Lecturer, Experimental and Clinical Oncology (516-635D), Department of Experimental Medicine, McGill University: Basic Research Issues in Lymphoma. |
| 2006-present | Lecturer, Post-Graduate Course: Integrated Systems Biology: BIM-6065, Hematopoietic Stem Cell Usage, University of Montreal. |

**POST-GRADUATE STUDENTS**:

| | | |
|---|---|---|
| 2002-present | Name: | Nadia Hebib (Post-Ph.D.) |
| | Title: | Induction of immune tolerance after allogeneic stem cell transplantation. |
| 2003-present | Name: | David Allen (Post-M.D.) |
| | Title: | Endothelial progenitor cells mobilization induced by hematopoietic growth factors. |
| 2004-present | Name: | Ahmed Ammar (post-MD): M.Sc. program) |
| | | Hematopoietic stem cells to repair trabecular cells |

**GRADUATE STUDENTS**:

| | | |
|---|---|---|
| 1990-1992 | Name: | Sylvie Arbour (M.Sc.) |
| | Title: | Anti-leukemia activity of bone marrow cells. |
| 1992-1997 | Name: | Marie-Hélène Lachapelle (Ph.D.) |
| | Title: | Endometrial natural killer cells. |
| 1993-1997 | Name: | Brigitte Léonard (M.Sc.) |
| | Title: | Evaluation of minimal residual disease using quantitative PCR. |
| 1995-2003 | Name: | Martin Guimond (Ph.D.) |
| | Title: | Graft-versus-leukemia effector cells. |
| 1996-2002 | Name: | Nadine Beauger (Ph.D.) |
| | Title: | Role of stem cell expansion for purging of malignant cells. |
| 1997-2003 | Name: | Brigitte Léonard (Ph.D.) |
| | Title: | Pathogenic role of the Bcl-2/IgH rearrangement. |
| 1998-2002 | Name: | Francis Parent (M.Sc.) |
| | Title: | Graft-versus-leukemia activity post-allogeneic transplantation for chronic myelogenous leukemia. |
| 1999-2002 | Name: | Céline Brazeau (M.Sc.) |
| | Title: | MDR-related transporters involved in preferential retention of rhodamine-derived photosensitizers. |
| 2000-2003 | Name: | Radia Sidi Boumédine (M.Sc.) |
| | Title: | Photodynamic prevention of graft-versus-host disease through selective elimination of alloreactive cells. |

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| 2001-2004 | Name:<br>Title: | Marie-Pier Giard (M.Sc.)<br>Ex vivo expansion of megakaryocytic progenitors for autologous stem cell transplantation. |
| 2002-2005 | Name:<br>Title: | Lynn Jaafar (M.Sc.)<br>Treatment of acute graft-versus host disease using a photopheresis approach |
| 2002-present | Name:<br>Title: | Pierre Fokam (M.Sc.)<br>Induction of an immunotolerance for the treatment of chronic graft-versus-host disease. |
| 2002-2006 | Name:<br>Title: | QinYong Dai (Ph.D.) (Experimental Medicine, McGill University)<br>Anti-tumor vaccination via a photodynamic approach. |
| 2004-present | Name:<br>Title: | Stéphanie Beauchemin (M.Sc.)<br>Ex vivo expansion of hematopoietic stem cells using the TAT-HOXB4 molecule. |
| 2004-present | Name:<br>Title: | Jean-Philippe Bastien (M.Sc.)<br>Induction of immunotolerance using photodynamic therapy. |
| 2005-present | Name:<br>Title: | Mireille Guérin (M.Sc.)<br>Anti-leukemia activity of PCK-3145. |
| 2005-present | Name:<br>Title: | Sabrina Vinet (M.Sc.)<br>Signaling potentiation to promote erythropoiesis. |
| 2007 | Name:<br>Title: | Véronique Gaudet (B.Sc. U. de M)<br>Evaluation des capacités migratoires de cellules souches hématopoïétiques murines dans un modèle de surexpression de la protéine HOX B4.<br>Biomédical Sciences– SBM 3008 (4 month) |
| 2007 – présent | Name :<br>Title : | Renaud Manuguerra-Gagné (M.Sc.)<br>Régénérescence des cellules trabéculaires d'un œil glaucomateux par des cellules souches mésenchymateuses. |
| 2007- présent | Name :<br>Title : | Marie Caudrelier (M.Sc.)<br>L'impact de la thérapie photodynamique sur les cellules dendétriques. |
| 2007- présent | Name :<br>Title : | Irina Cristina Helici (M.Sc.)<br>Effet de la protéine HOXB4 sur l'expansion et la différentiation des progéniteurs myéloïdes. |

**PARTICIPATION IN M.C. AND PH.D THESIS COMMITTEES AND COMPREHENSIVE EXAMS (PH.D) ( excluding jury of my own students)**

| | | |
|---|---|---|
| 1991 | Name : | Sylvie Fournier - Medicine/Biomedical Sciences |
| | Title : | Expression and regulation of the CD23 antigen on normal and malignant B cells. |
| | Role : | Member of Jury, Final Exam, Université de Montréal. |
| 1994 | Name: | Lidia Morelli, Ph.D. - Medicine/ Experimental Medicine |
| | Title : | Characterization of NK 1.2, an antigen with specific for murine NK cells. |
| | Role : | Member of Jury, Ph.D. Thesis, McGill University. |
| 1996 | Name : | Julio Robert Caceres Cortes, Ph.D. - Medicine/Biomedical Sciences |
| | Title : | The role of steel factor (SF) in the biology of human acute myeloblastic leukemia and normal CD34 + hematopoietic cells. |
| | Role : | Member of Jury, Ph.D. Thesis, Université de Montréal. |
| 1998 | Name : | Gael Dulude, Ph.D. - Medicine/Biomedical Sciences |
| | Title : | Thymic and extra-thymic T lymphocyte development in hematopoietic chimeras. |
| | Role : | Member of Jury, Final Exams, Université de Montréal. |
| 1998 | Name : | Stephane Pion, Ph.D. - Medicine/Biomedical Sciences |
| | Title : | Mechanisms of immunodominance. |
| | Role : | President of Jury, Ph.D. Thesis, Université de Montréal. |
| 1999 | Name : | Hashem Salloukh, Ph.D. - Medicine/ Experimental Medicine |
| | Title : | Genomic Instability in P190 Bcr-alb Leukemia Mouse Model. |
| | Role : | Member of Jury, Ph.D. Thesis, McGill University. |
| 2001 | Name : | Véronique Mateo, Ph.D. – Microbiology/Immunology |
| | Title: | Analysis of a novel form of cellular death induced by CD47 in lymphocytes. |
| | Role : | Member of Jury, Ph.D. Thesis, Université de Montréal. |
| 2001 | Name : | Julia M. Brain-Holcomb, Ph.D. – Medicine/ Experimental Medicine |
| | Title : | Genomic Instability in Chronic Myelogenous Leukemia. |
| | Role : | Member of Jury, Ph.D. Thesis, McGill University. |
| 2002 | Name : | Marie-Christine Meunier, M.Sc. - Medicine/Biomedical Sciences |
| | Title : | Treatment of leukemia using adoptive transfer of T lymphocytes specific for a minor histocompatibility antigen. |
| | Role : | President of Jury, Ph.D. Exam, Université de Montréal. |

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| 2004 | Name: | Robert Mio, Ph.D. – Médecine/sciences biomédicales |
| | Title: | Genetic-epidemiologic analysis of X-inactivation skewing in human females: suggestive evidence for two distinct traits |
| | Role: | Representative of Dean, Ph.D. Exam, Université de Montréal. |
| 2007 | Name : | Marie-Christine Meunier, Ph.D. – Médecine/sciences biomédicales |
| | Title : | Traitement du cancer par transfert adoptif de lymphocytes T dirigés contre un antigène mineur d'histocompatibilité. |
| | Role : | President of Jury, Thèse – Ph.D. |

**CLINICAL RESEARCH AND HOSPITAL SERVICE RESPONSIBILITIES:**

| | |
|---|---|
| 1990- present | Attending Physician, Bone Marrow Transplant Service, Maisonneuve-Rosemont Hospital |
| 1991- | Investigator, National Cancer Institute (USA), no 21418. |
| 1990-1995 | Co-Chairman, Phase I Cancer Clinical Research Protocol: Autologous Bone Marrow Transplantation for MY9 Positive Leukemia (Immunotoxins); Maisonneuve-Rosemont Hospital and Dana-Farber Cancer Institute, Boston MA |
| 1991-1993 | Principal investigator, Phase II Cancer Clinical Research Protocol: Emergency use of XomaZyme-CD5 Plus for the Treatment of Moderate to Severe Acute Graft versus Host Disease Unresponsive to Systemic Therapy with Steroids Maisonneuve-Rosemont Hospital-Xoma Corp. USA. |
| 1991-1995 | Co-Chairman, Phase I Cancer Clinical Research Protocol: Autologous Bone Marrow Transplantation for B4 Positive Leukemia (Immunotoxins); Maisonneuve-Rosemont Hospital and Dana-Farber Cancer Institute, Boston MA |
| 1991-1995 | Chairman, Phase I Cancer Clinical Research Protocol: Autologous Bone Marrow Transplantation with Anti-B4-bR Purging for Patients with Non Hodgkin's Lymphoma; Maisonneuve-Rosemont Hospital. |
| 1995-2000 | Chairman, Phase I Cancer Clinical Research Protocol: A Phase I Study of Bone Marrow Purging Using Photodynamic Therapy with Benzoporphyrin Derivative Monoacid Ring A in Patients with Acute Leukemia (HPB: Investigator IND). |
| 1995-2002 | Co-Investigator: Phase III Cancer Clinical Research Protocol: A Phase III Study of Radiotherapy or ABVD Plus Radiotherapy Versus ABVD Alone in the Treatment of Early Stage Hodgkin's Disease, Maisonneuve-Rosemont Hospital, NCIC CTG: HD.6. |
| 1996-1998 | Chairman, Phase I Cancer Clinical Research Protocol: Autologous CD34+ Stem Cell Transplantation for Patients with Chronic Myelogenous Leukemia, Maisonneuve-Rosemont Hospital. |
| 1997-2000 | Principal investigator; Phase II Cancer Clinical Research Protocol: A study of the Efficacy and Safety of CMA-676 as Single Agent Treatment of Patient with Acute Myeloid Leukemia (AML) in First Relapse; Maisonneuve-Rosemont Hospital. |

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| 1998-2003 | Chairman, Phase I Cancer Clinical Research Protocol: Autologous TH9402 Purged Stem Cell Transplantation for Patients with Chronic Myelogenous Leukemia, Celmed BioSciences - Maisonneuve-Rosemont Hospital. |
| 1999-present | Principal investigator; Phase III Cancer Clinical Research Protocol: A Phase III Study of STI571 versus Interferon-a (IFN-a) Combined with Cytarabine (Ara-C) in Patients with Newly Diagnosed Previously Untreated Philadelphia Chromosome Positive (Ph+) Chronic Myelogenous Leukemia in Chronic Phase (CML-CP); Maisonneuve-Rosemont Hospital. |
| 2000-present | Chairman, Phase I Cancer Clinical Research Protocol: Autologous TH9402 Purged Stem Cell Transplantation for Patients with Non-Hodgkin's Lymphoma, Celmed-BioSciences - Maisonneuve-Rosemont Hospital, and Chair for the multicenter study. |
| 2001-2002 | Co-Investigator, Phase II Cancer Clinical Research Protocol:: A Phase II Study of Troxatyl in Patients with CML Blastic Phase Disease, Biochem Pharma, Maisonneuve-Rosemont Hospital. |
| 2001-2002 | Co-Investigator; Phase I/II Cancer Clinical Research Protocol: A Phase I/II Multi-Center Study of Troxacitabine in Relapsed or Refractory Lymphoproliferative Neoplasms or Multiple Myeloma (BCH-4556-211-A5), Shire Pharmaceutical Development, Hôpital Maisonneuve-Rosemont. |
| 2002-2004 | Co-Investigator; Phase III Cancer Clinical Research Protocol, Intergroup Trial of First Line Treatment for Patients with Diffuse Large B-Cell Non-Hodgkin's Lymphoma with a CHOP-like Chemotherapy Regimen with or without the anti-CD20 Antibody Rituximab, The Mabthera International Trial (MINT) Study Group; NCIC CTG: LY.9, Hôpital Maisonneuve-Rosemont. |
| 2002- present | Principal Investigator; Phase III Cancer Clinical Research Protocol, A Randomized Phase III Trial Comparing Early High Dose Chemoradiotherapy and an Autologous Stem Cell Transplant to Conventional Dose CHOP Chemotherapy (With Possible Late Autologous Stem Cell Transplant) for Patients With Diffuse Aggressive Non-Hodgkin's Lymphoma in the High-Intermediate and High Risk International Classification Prognostic Groups. SWOG; NCIC CTG: LY.11, Hôpital Maisonneuve-Rosemont. |

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| 2002-2004 | Co-Investigator; A Randomized Phase I Study of Two Different Schedules of BAY 43-9006 in Patients With Acute Myeloid Leukemia or Myelodysplastic Syndrome; NCIC CTG: IND.152, Maisonneuve-Rosemont Hospital. |
| 2002- present | Co-Investigator; Efficacy and Safety of Subsequent Treatment with $^{90}$Y-Ibritumomab Tiuxetan Versus No Further Treatment in Patients with Stage III or IV Follicular Non-Hodgkin's Lymphoma Having Achieved Partial or Complete Remission After First Line Chemotherapy. A Prospective, Multicenter, Randomized Phase III Clinical Trial; Berlex, Maisonneuve-Rosemont Hospital. |
| 2002- present | Co-Investigator; A Phase II Study of PS-341 (NSC 681239) in Patients with Untreated or Relapsed Waldenstrom's Macroglobulinemia; NCIC CTG: IND.152, Maisonneuve-Rosemont Hospital. |
| 2003- 2006 | Chairman; Phase I: A Dose-Escalation Study of PBI-1402 and its Effect on Human Hematopoietic Cell Populations in Healthy Human Volunteers, ProMetic Biosciences - Maisonneuve-Rosemont Hospital. |
| 2003- present | Chairman, Phase I/II: Haploidentical peripheral blood stem cell transplantation with CD34+ cell selection and a T-cell add-back depleted of host alloreactive cells using photodynamic cell therapy, Celmed BioSciences – Maisonneuve-Rosemont Hospital. |
| 2004-present | Co-Investigator; Protocol R115777-AML-301; Phase III. A randomized study of tipifarnib versus best supportive care (including hydroxyurea) in the treatment of newly diagnosed acute myeloid leukemia (AML) in subjects 70 years or older; Johnson & Johnson Pharmaceutical Research & Development, Maisonneuve-Rosemont Hospital. |
| 2005-present | Investigateur principal; Protocole Phase III Cancer Clinical Research Protocol: An Open-Label, Randomized, Multicentre Study Comparing Gleevec (Imatinib) at Doses of 400 mg and 800 mg in Patients with Chronic-Phase Philadelphia Chromosome-Positive Chronic Myeloid Leukemia Who Have Reached Complete Cytogenetic Response with Gleevec® (imatinib) 400 mg. GLEEM (CSTI571ACA09), Novartis, Hôpital Maisonneuve-Rosemont. |
| 2005-present | Principal Investigator; Phase I/II Cancer Clinical Research Protocol: A phase IA/II multicenter, dose-escalation study of oral AMN107 on a continuous daily dosing schedule in adult patients with Gleeved-Resistant CML in accelerated phase or blast crisis, relapsed/refractory Ph+ All, and other hematologic malignancies, (CAMN107A2101), Novartis, Hôpital Maisonneuve-Rosemont. |

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| 2005- present | Co-Investigator, Phase III Cancer Clinical Research Protocol: A double-blind, randomized, placebo-controlled, parallel-group study to evaluate the efficacy, safety, tolerability, pharmacokinetics and pharmacodynamics of SB 497115-GR, a thrombopoietin receptor agonist, administered as once-daily 30-, 50- and 75-mg tablets for six weeks to adults with refractory, chronic immune thrombocytopenic (ITP). Protocol TRA100773 Glaxo SmithKline, Hôpital Maisonneuve-Rosemont. |
| 2005- present | Co-investigator; Phase III Cancer Clinical Research Protocol: Phase III clinical study of allogeneic stem cell transplantation with reduced intensity conditioning (RICT) versus best standard of care in acute myeloid leukemia (AML) in first complete remission. Protocol TRALG 1/02 CBMTG, Hôpital Maisonneuve-Rosemont. |
| 2005- present | Co-investigator, Phase III Cancer Clinical Research Protocol: An open-label, randomized, phase 3 trial of intravenous temsirolimus (CCI-779) at two dose levels compared to investigator's choice therapy in relapsed, refractory subjects with mantle cell lymphoma (MCL). Protocol 3066K1-305-WW Wyeth Research, Hôpital Maisonneuve-Rosemont. |
| 2006-present | Co-Principal Investigator ; Phase III Cancer Clinical Research Protocol: A randomized, controlled, parallel-group, multicenter study of ExtraCorporeal Photoimmune Therapy with Therakos UVADEX for the treatment of patients with newly diagnosed Acute GvHD. (Acute GvHD-1), Therakos inc., Hôpital Maisonneuve-Rosemont |
| 2006-present | Principal Investigator; Phase Ib Cancer Clinical Research Protocol: Effect of PBI-1402 in cancer patients undergoing chemotherapy and suffering from anemia. (PBI-251005) ProMetic BioSciences Inc., Hôpital Maisonneuve-Rosemont. |
| 2006-present | Co-Investigator; Phase III Cancer Clinical Research Protocol: A Phase III Randomized, Double-Blind, Controlled Study Comparing Clofarabine and Cytarabine versus Cytarabine Alone in Adult Patients ≥60 Years Old with Acute Myelogenous Leukemia (AML) who have Relapsed or are Refractory after Receiving up to Two Prior Induction Regimens, Genzyme Corp., Hôpital Maisonneuve-Rosemont. |
| 2007-présent | Co-investigator, An open-label, randomized, phase 3 study of inotuzumab ozogamicin (CMC-544) administered in combination with Rituximab compared to a defined investigator's choice therapy in subjects with relapsed or refractory, CD22-positive, follicular B-cell, Non Hodgkin's lymphoma. CMC-544 Protocol no. 3129K4-3301-WW (Wyeth Research), Hôpital Maisonneuve-Rosemont. |

**Interview with Media:**

- Report in La Presse (Pascale Breton, 2007)
- Interview, September 2007, « Une pilule, une petite granule » (Télé Québec, October 2007)
- Interview with Ms Louise Deschatelets (Canal Vox, April 3rd, 2007) (1 hour)
- Interview with Mr Paul Arcand (98,5 FM, February 22nd, 2007) (1 hour)
- Interview with Dominique Poirier (RDI, January 8th, 2007)
- Report from Esther Normand (Radio-Canada, January 8th, 2007)
- Report from Émilie Perreault (TQS, January 8th, 2007)
- Interview at 940 AM (Charles Pitts, September 15th, 2006)
- Interview at CKAC (Charles Pitts, September 9th, 2006)
- Report in Le Devoir (Pauline Gravel « Un centre de recherche sur les cellules souches ouvrira à Montréal », September 7th, 2006)
- Report in Le Journal de Montréal (Éric-Yvan Lemay, « La voie de l'avenir », September 7th, 2006)
- Report in La Presse (Pascale Breton, « Les cellules souches cultivées sont sources d'espoir », September 7th, 2006)
- Report in The Gazette «(Aaron Derfel, « Maisonneuve-Rosemont hospital to build stem-cell centre », September 7th, 2006)
- Interview with Anne-Sophie Hennekens (Radio-Canada, September 7th, 2006)
- Reportage à Radio-Canada (Esther Normand, September 6th, 2006)
- Reportage à TVA (Maryse Gagnon, September 6th, 2006)
- Entrevue à Dominique Poirier en direct (RDI, Septembre 6th, 2006)

**PATENTS**

1. P. Miron, M. H. Lachapelle, **D. C. Roy**. Use of Ligands Specific to Major Histocompatibility Complex Class 1 Antigens for Diagnosing Endometriosis. USA Patent: N.S. 08/0860, 064. International Patent no. PCT/CA95/00730.

2. **D. C. Roy**, M. Guimond, N. Molfino. Rhodamine derivatives for photodynamic diagnosis, prevention and treatment of immunologic disorders. USA patent: 65/157,790. European Patent 126793.

3. **D. C. Roy**, L., Villeneuve, M. Vaillancourt. PDT-Treated Cells or Lysate thereof for Preparation of an Autoimmune Vaccine for Immunological Disorders and Cancers. US-Pr107-03.

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**A) Original Articles:**


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2. **Roy, D.C.,** et Couture, J. Functional residual capacity and lung mechanics study with curarization and after anesthesia. Rapport des travaux scientifiques, programme des stagiaires de la Faculté de Médecine, Presses de l'Université de Montréal 1979, 3:1-15.

3. **Roy, D.C.**, Havrankova, J., Belanger, R, Pomier, G., Tanguay, S., D'Amour, P., et Matte, R. Localization of insulinomas: Evaluation of transhepatic catheterization of pancreatic venous system with insulin radioimmunoassey. Union Méd. Can. 1985, 114:415-420.

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7. **Roy, D.C.**, Griffin, J.D., Belvin, M., Blättler, W.A., Lambert, J.M. and Ritz, J. Anti-MY9-Blocked-Ricin: An Immunotoxin for Selective Targeting of Acute Myeloid Leukemia Cells. Blood, 1991, 77:2404-2412.

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111. Bastien J.P., Giard, M.P., Krosl, G., **Roy, D.C**. Le traitement photodynamique de la maladie du greffon contre l'hôte au TH9402 permet d'éliminer les cellules T alloréactives tout en conservant les cellules T régulatrices. 13rd Annual Research Day, Maisonneuve-Rosemont Hospital, Montreal, June 2006.

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116. Guérin, M., Thérien, C., **Roy, D.C**. Le traitement des cancers hématologiques par le PCK3145, un peptide de 15 acides aminés dérivés de la protéine spécifique de la prostate PSP-94. 14th Annual Research Day, Maisonneuve-Rosemont Hospital, Montreal, June 1st, 2007.

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121. Bijl, J., Cherief, H., **Roy, D.C.** Deletion of HoxAGenes inhibits proliferation and differentiation of myeloid progenitors. 49th Annual Meeting of the American Society of Hematology, Atlanta, GA, December 8-11th. Blood 2007, vol 110, no 11, 1271.

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127. Bastien, J.P., Krosl, G., Dubé, P., Therien, C., Scotto, C., D.C. Roy. Regulatory T cells spared by photodynamic therapy of cells from chronic graft-versus-host disease patients inhibit alloreactivity. 11th Biennial Conference of the Canadian Blood and Marrow Transplant, 9-12 avril 2008, Montreal, Québec.

**D) Audiovisual document:**

R.D. Gascoyne, R. Fisher, J.G. Gribben, **D.C. Roy.** Indolent B-Cell Lymphoma: State of the Art. CD-ROM interactif subventionné par Berlex Canada, CD-Pharma Interactive Medical Productions, 2000.

R. Gascoyne, T. Kouroukis, T.E. Witzig, H. Veelken, P. Laneuville, **D.C. Roy.** Montreal Symposium on Lymphoma. CD-ROM interactif produit par le Groupe d'Etude en Oncologie du Québec (GEOQ), 2003.

**E) Invited lectures:**

1. **Roy, D.C.** Autologous Bone Marrow Transplantation with Immunological Purging of Malignant Cells. Hematology Day of Notre-Dame Hospital/Adria, Montreal, Que., September 1989.

2. **Roy, D.C.** Is Cure Possible in Refractory Lymphomas? Experience of the Dana-Farber Cancer Institute. Hematology Day of Notre-Dame Hospital/Adria, Montreal, Que., Sept. 1989.

3. **Roy, D.C**: Bone Marrow Transplantation: Immunological Purging Methods. Montreal Red-Cross Research Symposium, Montreal, November 1989.

4. **Roy, D.C.** Immunological Methods Used to Eliminate Malignant Cells from a Marrow Graft. Symposium of the Quebec Association of Microbiologists, Montreal, February 1990.

5. **Roy, D.C.** Immunologic Investigation in Oncology. XIIth Immunology Seminar of the University of Montreal, Montreal, April 1990.

6. **Roy, D.C.** Autologous Bone Marrow Transplantation for acute leukemia. Hematology, Sainte-Justine Hospital, April 20th, 1990.

7. **Roy, D.C.** Immunotoxins in bone marrow transplantation. Radiotherapy Department, Notre-Dame Hospital, October 11th, 1990.

8. **Roy, D.C.** Novel developments in autologous and allogeneic bone marrow transplantation. Medical Grand Rounds, Hôpital de l'Enfant-Jésus, Quebec City, November 8, 1990

9. **Roy, D.C.** Autologous Bone Marrow Transplantation for Acute Leukemia. XVIth Annual Meeting of the Medical Biologists of the Province of Quebec, Trois-Rivières, March 24th, 1991.

10. **Roy, D.C.** Monoclonal Antibodies for Depletion of Autologous Bone Marrow Grafts. C.P.T.M.Q. and S.C.T.I. Annual Meeting, Montreal, May 1991.

11. **Roy, D.C.** Immunotoxins in Bone Marrow Transplantation. Symposium on Organ Transplantation, Montreal, Oct. 24th, 1991.

12. **Roy, D.C.** New Developments in Autologous Bone Marrow Transplantation. Joint Meeting of Canadian Society for Transfusion Medicine/Canadian Red Cross Society, Winnipeg, Manitoba, May 29th, 1992.

13. **Roy, D.C.** Autologous Bone Marrow Transplantation. XVIIth Annual Meeting of the Quebec Association of Hematologists-Oncologists, Val David, June 11th, 1992.

14. **Roy, D.C.** Bone Marrow Transplantation : Who ? When ? Why ? Hematology in General Practice, Hôtel Radisson Gouverneurs, Montreal, November 13th, 1992.

15. **Roy, D.C.** Acute Myeloid Leukemia Treated by Autologous Purged Marrow Transplantation. Medical Grand Rounds, Hôpital du Saint-Sacrement, Québec, 5 February 5th, 1993.

16. **Roy, D.C.** Update in Bone Marrow Transplantation. Medical Grand Rounds, Hôpital de Chicoutimi, February 18th, 1993.

17. **Roy, D.C.** Immunotoxins in Hematologic Malignancies. Conference of the Department of Pharmacology, Université de Montréal, March 19, 1993.

18. **Roy, D.C.** Immunotherapy in Bone Marrow Transplantation. Hematology Group of the Montérégie, Carignan, June 3, 1993.

19. **Roy, D.C.** Autologous Purged Bone Marrow Transplantation for Non Hodgkin's Lymphoma. Low Grade Non-Hodgkin's Lymphoma: A Canadian Consensus Conference. Toronto, Ontario, September 20th, 1994.

20. **Roy, D.C.** Autologous anti-B4-bR Purged Bone Marrow Transplantation for the Treatmen of Non-Hodgkin's Lymphoma. Lymphoma Club, McGill University, October, 1994.

21. **Roy, D.C.** High Dose Therapy in Non-Hodgkin's Lymphoma with Immunotoxin Purging. Fourth International Meeting of the Canadian Bone Marrow Transplantation Group. Ottawa, Ontario, November 5th, 1994.

22. **Roy, D.C.** Intensive Therapy for Non-Hodgkin's lymphoma. Meeting of Oncopharmacists of Quebec - SPOC, Montreal, March 16, 1995.

23. **Roy, D.C.** Minimal Residual Disease in Non-Hodgkin's Lymphoma. Montreal Cancer Research Group Meeting, McGill University, March 26th, 1996.

24. **Roy, D.C.** Low Grade Non-Hodgkin's Lymphomas. Meeting of Oncopharmacists of Quebec - SPOC, Montreal, November 9th, 1996.

25. **Roy, D.C.** Clinical Aspects of Flow-Cytometry Analysis of Progenitor Cells. Flow-Cytometry Symposium - Coulter Immunology, Montreal, November 20th, 1996.

26. **Roy, D.C.** Practical Approach to Clinical Studies in Oncology. Canadian Cancer Society Montreal, Nov. 27, 1996.

27. **Roy, D.C.** New Approaches to Purging. Frontiers of Malignant Hematology. Toronto, Ontario, June 7th, 1997.

28. **Roy, D.C.** Role of Autografting in the Management of Acute Myeloid Leukemia. Sixth Canadian Bone Marrow Transplant Group Meeting, Vancouver, BC, March 27th, 1998.

29. **Roy, D.C.** New Developments in the Treatment of Hematopoietic Cell Grafts. Annual Meeting of Professional Medical Technologists of Quebec, St-Hyacinthe, Quebec, May 29th, 1998.

30. **Roy, D.C.** Novel Strategies in Autologous Stem Cell Transplantation. Medical Grand Rounds, Hôpital Charles-Lemoyne, Greenfield Park, Quebec, February 4th, 1999.

31. **Roy, D.C.** The Role of Purging: Why should we purge? The 7th Canadian Bone Marrow Transplant Group Meeting, Québec, April 8, 2000.

32. **Roy, D.C.** New Developments in Hematopoietic Stem Cell Transplantation. Medical Grand Rounds, Centre Hospitalier Régional de Lanaudière, Joliette, April 26th, 2000.

33. **Roy, D.C.** Critique of the "Treatment of Elderly Patients with Aggressive Histology Lymphoma: Cancer Care Ontario Practice Guideline". Satellite symposium of The National Cancer Institute of Canada (NCIC-CTG), Spring Meeting, Toronto, May 2000.

34. **Roy, D.C.** Immunologic Mechanisms of Monoclonal Antibodies. Meeting of the Association of Pharmacists of Health Institutions of Quebec (APES), Hôtel des Gouverneurs, Montreal, Nov. 23, 2001.

35. **Roy, D.C.** Improving Stem Cell Transplantation through Manipulation of the Graft. Jewish General Hospital Medical Grand Rounds, Montreal, March 18th, 2002.

36. **Roy, D.C.** What is State-of-the-Art Stem Cell Transplantation ? North-Shore Hematology-Oncology Group, Laval, March 19th, 2002.

37. **Roy, D.C.** Use of Light to Eliminate Alloreactive T Lymphocytes and Tumor Cells. National Marrow Donor Program Annual Council Meeting, Minneapolis, Minessota, USA, October 12th, 2002.

38. **Roy, D. C.** Visible Light to Eliminate Cancer Cells and Immunoreactive T Cells. Department of Hematology / Oncology, University Medical Center, Freiburg, Germany, November 25th, 2002.

39. **Roy, D. C.** Lights on Allogeneic and Autologous Stem Cell Transplantation. Fred Hutchinson Cancer Research Center Grand Rounds, Seattle, USA, June 10, 2003.

40. **Roy, D.C.** Overview of CML treatment. Practice guidelines for the treatment of patients with Chronic Myeloid Leukemia: Canadian Recommendations 2004, Montreal, Canada, October 1st, 2004.

41. **Roy, D.C.** Radio-immunotherapy: A new approach to treat lymphomas. Interdisciplinary Oncology/Nuclear Medicine Meeting, Montreal, June 2nd, 2005.

42. **Roy, D.C.** Oxydative stress in the clinical setting: Deleterious or beneficial? 12th Research Day, Hôpital Maisonneuve-Rosemont Research Day, Montreal, June 3rd, 2005.

43. **Roy, D.C.** Indications and integration of radioimmunotherapy in the treatment of follicular lymphomas. Symposium on lymphomas, Carling Lake, Quebec, October 2nd, 2005.

44. **Roy, D.C**. Cellular therapy: an approach to expand cells and indications for transplantation. University of Montreal, Roger-Gaudry Pavilion, Montreal, November 24th 2005.

45. **Roy, D.C.** Photodynamic Therapy a Useful Adjunct to the Treatment of Hematologic Malignancies. Montreal General Hospital Hematology Research Rounds, Montreal, January 18th, 2006.

46. **Roy, D.C**. Update on New Tasigna data (Chronic and Advanced Phases). CML Meeting Mont Tremblant, April 1st, 2007.

47. **Roy, D.C.** Radioimmunotherapy (RIT) of LNH low grade recidivants. Annual Meeting of the Association of Specialists in Nuclear Medicine, Hotel Le Montagnais, Chicoutimi, April 14th, 2007.

48. **Roy, D.C**. Workshop : Stem cells : from benchtop to bedside. Recombinant HOXB4 for human stem cell expansion. Montreal Neurologic Institute, Montréal, Québec, June 4th, 2007.

49. **Roy. D.C.** Lights on photodynamic therapy. Ohio State University Grand Rounds, Columbus, OH, USA, April 2008.

**F) Session Chair (Meetings or Symposia):**

1. **Roy, D.C.** Plenary Session Chair: In vitro treatment of bone marrow grafts. Fourth International Meeting of the Canadian Bone Marrow Transplantation Group. Ottawa, Ontario, November 5th, 1994.

2. **Roy, D.C.** Plenary Session Chair: Basic Science and Future Directions in Acute Leukemia Therapy. Frontiers of Malignant Hematology. Toronto, Ontario, June 7th, 1997.

3. **Roy, D.C.** Plenary Session Chair: Myelodysplastic syndromes, Frontiers of Malignant Hematology-II. Montreal, June 19th, 1999.

4. **Roy, D.C.** Session Chair: Update in indolent B cell lymphomas, Berlex Satelite Hematology/Oncology Symposium. Quebec, May 4th, 2000.

5. **Roy, D.C.** Session Chair: Improving survival in follicular lymphoma. Myth or reality?, Berlex Canada Oncology Symposium. Montreal, October 23rd, 2000.

6. **Roy, D.C.** Session Chair : Cell Processing – Purging and Depletion, 42nd Annual Meeting of the American Society of Hematology (ASH). San Francisco, CA, December 4th, 2000.

7. **Roy, D.C.** Session Chair: "Seminar on lymphomas", Symposium of the Quebec Oncology Study Group (GEOQ). Quebec City, February 9th, 2002.

8. **Roy, D.C.**: Plenary Session Chair: Haploidentical transplants. Eight Biannual Conference of the Canadian Bone Marrow Transplantation Group. Halifax, Nova-Scotia, June 1st, 2002.

9. **Roy, D.C.** Session Chair: Symposium on Hematological Manipulations to Protect Neural Cells. Ortho-Biotech – International Society of Experimental Hematology Annual Meeting. Montreal, Quebec, July 6th, 2002.

10. **Roy, D.C.** Session Chair: Leukemia - Clinical. 31st Annual Meeting of the International Society of Experimental Hematology. Montreal, Quebec, July 7th, 2002.

11. **Roy, D.C.** Plenary Session Chair: Marrow and Blood Stem Cell Transplantation. 31st Annual Meeting of the International Society of Experimental Hematology. Montreal, Quebec, July 7th, 2002.

12. **Roy, D.C.** Session Chair: Symposium on the treatment of lymphomas. Montreal, Canada, September 26th, 2003.

13. **Roy, D.C.** Session Chair: Clinical Results - Alternative Donor Transplantation: Reduced Intensity / Non-Malignant, 42nd Annual Meeting of the American Society of Hematology. Atlanta, GA, December 12th, 2005.

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# High incidence of leukemia in large animals after stem cell gene therapy with a *HOXB4*-expressing retroviral vector

Xiao-Bing Zhang,[1] Brian C. Beard,[1] Grant D. Trobridge,[1,2] Brent L. Wood,[3] George E. Sale,[1] Reeteka Sud,[1] R. Keith Humphries,[4] and Hans-Peter Kiem[1,2]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA. [2]Departments of Medicine and Pathology and [3]Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA. [4]Terry Fox Laboratory, British Columbia Cancer Agency, and Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada.

**Retroviral vector–mediated HSC gene therapy has been used to treat individuals with a number of life-threatening diseases. However, some patients with SCID-X1 developed retroviral vector–mediated leukemia after treatment. The selective growth advantage of gene-modified cells in patients with SCID-X1 suggests that the transgene may have played a role in leukemogenesis. Here we report that 2 of 2 dogs and 1 of 2 macaques developed myeloid leukemia approximately 2 years after being transplanted with cells that overexpressed homeobox B4 (HOXB4) and cells transduced with a control gammaretroviral vector that did not express HOXB4. The leukemic cells had dysregulated expression of oncogenes, a block in myeloid differentiation, and overexpression of HOXB4. *HOXB4* knockdown restored differentiation in leukemic cells, suggesting involvement of HOXB4. In contrast, leukemia did not arise from the cells carrying the control gammaretroviral vector. In addition, leukemia did not arise in 5 animals with high-level marking and polyclonal long-term repopulation following transplantation with cells transduced with an identical gammaretrovirus vector backbone expressing methylguanine methyltransferase. These findings, combined with the absence of leukemia in many other large animals transplanted with cells transduced with gammaretroviral vectors expressing genes other than *HOXB4*, show that HOXB4 overexpression poses a significant risk of leukemogenesis. Our data thus suggest the continued need for caution in genetic manipulation of repopulating cells, particularly when the transgene might impart an intrinsic growth advantage.**

## Introduction

Gene therapy holds great promise for providing new treatments for a large number of genetic and acquired diseases (1), and retroviruses are currently the most efficient vectors for gene delivery to HSCs. Gammaretroviral vectors have shown early clinical success for X-linked SCID (SCID-X1) (2–5), adenosine deaminase SCID (ADA-SCID) (6–8), and X-linked chronic granulomatous disease (X-CGD) (9). However, 4 SCID-X1 patients developed T cell leukemia after gene transfer of IL2Rγ via an MLV-based gammaretroviral vector into HSCs 2–3 years after treatment, but not in other gene therapy trials (10, 11). These findings led to the notion that leukemogenesis caused by retroviral vectors may be associated with particular transgenes.

Studies have been carried out in mouse models to address safety issues involved in retroviral vector–based stem cell gene therapy (12–14). However, findings in murine models may not always clearly predict outcomes in a clinical setting, likely because of intrinsic differences between humans and mice. In addition, much higher stem cell doses are typically employed in clinical trials, resulting in the infusion of significantly higher numbers of gene-modified stem cells compared with mouse studies. In concert with this, studies in large animals like dogs and nonhuman primates have been more predictive than mouse studies. Thus, we have used large animal models to study HSC gene transfer to address the safety of gene-modified HSCs. We have reported that long-term analysis of large animals (17 dogs and 23 baboons) that received gene-modified cells identified no vector-mediated malignancies (15). More than 40 animals have now been followed a median time of 3.1 years and have maintained normal hematopoiesis.

Accumulating evidence from mouse studies suggests that homeobox B4 (HOXB4) overexpression enhances in vivo (16, 17) and ex vivo expansion of HSCs (18, 19). In contrast to the strong leukemogenic capacity of other members of the *HOX* gene clusters (20), HOXB4-expanded HSCs retain their normal differentiation and long-term repopulation potential, and no hematologic abnormalities have been detected in large groups of mice that were transplanted with HOXB4-transduced HSCs (16, 18). Our previous study in nonhuman primates showed that HOXB4 overexpression significantly expands repopulating cells, in particular short-term repopulating cells (21).

Here we characterized the leukemias that developed in the 3 animals that received HOXB4-transduced CD34$^+$ cells approximately 2 years after transplantation. We compared the findings to a control group of animals that received transduced CD34$^+$ cells with the same vector backbone but expressing the methylguanine methyltransferase (MGMT) gene.

## Results

*Development of leukemia in 3 animals approximately 2 years after transplantation.* We investigated the effects of HOXB4 overexpression in the dog and nonhuman primate models. Our studies show-

**Nonstandard abbreviations used:** HOXB4, homeobox B4; MGMT, methylguanine methyltransferase; MSCV, murine stem cell virus; PRDM16, PR domain containing 16; TSS, transcription start site; YFP, yellow fluorescent protein.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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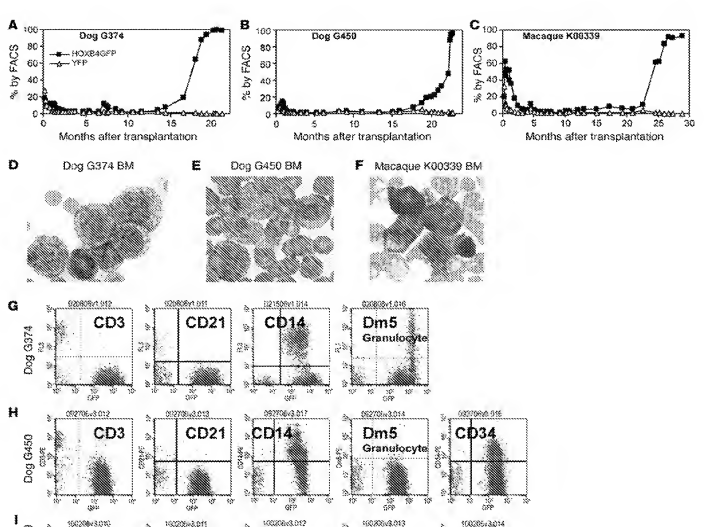


**Figure 1**

Development of leukemia in 2 dogs and 1 macaque transplanted with HOXB4-overexpressing CD34+ cells. Marking levels in granulocytes in dogs G374 (**A**) and G450 (**B**) and macaque K00339 (**C**). Representative marrow morphology at necropsy for G374 (**D**), G450 (**E**), and K00339 (**F**). High numbers of blast cells were observed in the marrow. Subset analysis of marrow samples from G374 (**G**), G450 (**H**) and K00339 (**I**). After red blood cell lysis, nucleated marrow cells were stained with antibodies to the T cell marker CD3, B cell marker CD20 or CD21, monocyte marker CD14, granulocyte marker Dm5 or CD13, and stem/progenitor cell marker CD34.

ing the effect of HOXB4 on hematopoietic repopulating cells in nonhuman primates have been previously published (21). Similar to our findings in nonhuman primates, we observed higher levels of HOXB4-overexpressing cells early after transplantation compared with levels after 1 month in dogs (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI34371DS1). One of the 3 dogs that received *HOXB4*-transduced CD34+ cells died 3 months after transplantation due to transplantation-related complications. The other 2 dogs (G374 and G450) were followed for 2 years. Approximately 15 months after transplantation, we observed a spontaneous increase of HOXB4-marked cells in dogs G374 and G450, while control yellow fluorescent protein–marked (YFP-marked) cells gradually decreased to <0.1% (Figure 1, A and B). The same pattern was seen in macaque K00339 at 22 months after transplantation (Figure 1C).

Dog G374 developed an increased white blood cell count with an increase in monocytes and a decrease in platelet count (Supplemental Figure 2). Bone marrow analysis confirmed the diagnosis of acute myelomonocytic leukemia (Figure 1D). Dog G450

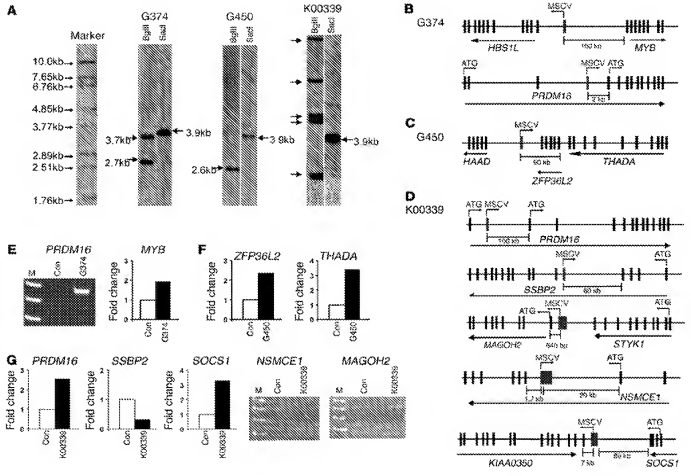


**Figure 2**

Retroviral integration induced mutagenesis is associated with leukemogenesis. (**A**) Southern blot analyses of marrow samples from G374, G450, and K00339 demonstrate monoclonality. Marrow DNA was digested with BglII, which cuts the transgene once, releasing a unique band for each integrant. Digestion with SacI, which cuts the transgene twice, showed a 3.9-kb band for all the integrants. (**B**–**D**) Integration sites were determined by LAM-PCR, and the schematic representations of integration sites for G374 (**B**), G450 (**C**), and K00339 (**D**) are shown. Exons are represented by black boxes. MSCV indicates the integration site, and the arrow indicates the orientation. ATG denotes the translation start site. Note that exons and introns are not to scale. (**E**–**G**) Dysregulated expression of genes in close vicinity of the integration sites for G374 (**E**), G450 (**F**), and K00339 (**G**). SYBR Green real-time RT-PCR was performed to determine the expression levels of dog *MYB* (**E**), dog *ZFP36L2* and thyroid adenoma associated (*THADA*) (**F**), and macaque *PRDM16*, *SSBP2*, and *SOCS1* (**G**). Canine *PRDM16* (**E**) and macaque *MAGOH2* (**G**) expression was undetectable in samples from normal control animals. (**G**) Expression of *NSMCE1* was undetectable in K00339 and control animals. Marrow mRNA samples from normal animals were used as controls. M, DNA ladder.

developed pancytopenia and an increased number of leukemic blast cells in the peripheral blood 20 months after transplantation (Supplemental Figure 3). Bone marrow and peripheral blood analysis confirmed the development of acute myeloid leukemia (AML) (Figure 1E).

Twenty-five months after transplantation, macaque K00339 developed a decreased hematocrit, an increase in nucleated red blood cells, and eventually an increase in white blood cells with circulating blasts (Supplemental Figure 4). Marrow analysis confirmed AML (Figure 1F).

All the animals were euthanized due to deteriorating physical condition associated with leukemia. Pathological examination of bone marrow and other tissues confirmed the diagnoses of leukemia. Leukemic blasts from G450 and K00339 expressed CD34 (Figure 1, G–I), whereas only about 1% of blasts were $CD34^+$ in G374. However, a leukemic cell line derived from G374 expressed CD34 (see Figure 3C).

Taken together, we observed the development of leukemia in 3 animals. In all 3 cases the leukemia arose in HOXB4GFP-expressing cells but not in YFP-expressing cells, suggesting that HOXB4 is the major contributor to the leukemia ($P = 0.05$).

*Gammaretrovirus integration induced mutagenesis is associated with leukemogenesis.* To examine the clonality of the leukemia, we performed Southern blot analyses. In G374, we detected 2 bands with the same intensity, suggesting 1 clone with 2 integration sites (Figure 2A). Analysis of DNA samples from different time points after transplantation confirmed this result (Supplemental Figure 5). In G450, we detected 1 band, demonstrating 1 clone with 1 integration site. DNA from K00339 showed 5 bands with the same intensity, suggesting 1 predominant clone with 5 inte-

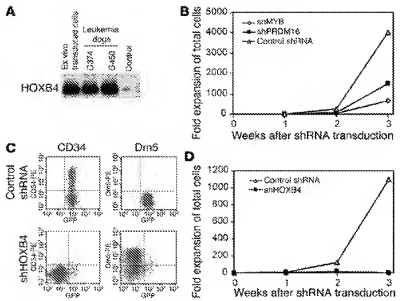


**Figure 3**
HOXB4 overexpression contributes to leukemogenesis. (**A**) Western blot analysis showed strong HOXB4 expression in marrow cells from leukemic animals. HOXB4-overexpressing dog cells in ex vivo culture were used as a positive control and marrow cells from normal dog as a negative control. (**B**) Knockdown of *PRDM16* or *MYB* decreased the proliferation rate of the dog G374 leukemic cells. (**C**) *HOXB4* knockdown in the leukemic cell line induced cell differentiation and (**D**) abolished cell proliferation. The stem cell marker CD34 and mature granulocyte marker Dm5 were used to evaluate cell differentiation. The leukemic cell line was cultured in medium supplemented with cSCF, thrombopoietin, and Flt3-L each at 100 ng/ml.

gration sites. PCR analysis of each integrant using DNA from a single colony derived from K00339 bone marrow confirmed this interpretation (data not shown).

Integration sites were determined by LAM-PCR (22). In dog G374, the vector was integrated at 100 kb upstream of the *c-myb* transcription start site (TSS) in the reverse orientation, and a second provirus was integrated into intron 3 of PR domain containing 16 (*PRDM16*) in the same orientation (Figure 2B). In G450, the vector was integrated at 90 kb downstream of *ZFP36L2* TSS (Figure 2C). Of interest, we also observed 1 integration site at *PRDM16* intron in K00339 (Figure 2D). Two integrants in this animal were at introns of single-stranded DNA binding protein 2 (*SSBP2*) and nonstructural maintenance of chromosomes element 1 homolog (*NSMCE1*) in reverse orientation, and 2 other integrants were at 640 bp upstream of mago-nashi homolog 2 (*MAGOH2*) and 60 kb downstream of *SOCS1* TSS (Figure 2D).

We next explored whether vector integration at oncogenes resulted in altered oncogene expression using real-time RT-PCR. In marrow from G374, expression of *PRDM16* was detected, whereas in normal control samples *PRDM16* was undetectable (Figure 2E). Further analysis demonstrated that the 5′ LTR was spliced into exon 4, activating the short isoform *PRDM16* (Supplemental Figure 6), which lacks the PR domain and has been reported to play an important role in leukemogenesis (23). In addition, *MYB* expression was also upregulated.

In G450, integration at the *ZFP36L2* locus was associated with increased expression of *ZFP36L2* and thyroid adenoma associated (*THADA*) expression compared with control bone marrow cells (Figure 2F).

In K00339 bone marrow, we detected increased expression of *PRDM16* and *SOCS1* and decreased expression of *SSBP2*, a tumor suppressor (Figure 2G). Given that *SOCS1* expression has been reported to be downregulated in many leukemic cells (24), it may suggest that the integration site at the *SOCS1* locus has not contributed to leukemia. Expression of *NSMCE1* was undetectable in all the samples, suggesting that it is a "passenger" integration site. Virus integration upstream of the TSS also appeared to activate *MAGOH2* expression; however, its function in mammalian cells is largely unknown. Taken together, *PRDM16* upregulation and SSBP2 downregulation, among other dysregulated genes, likely contributed to the development of leukemia in this animal.

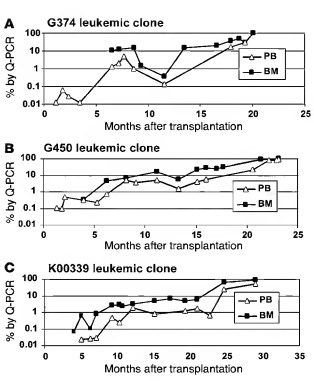


**Figure 4**
Increase in relative marking of leukemic clones over time. Real-time PCR was performed for dogs G374 (**A**) and G450 (**B**) and macaque K00339 (**C**) using LTR- and chromosome-specific primers. Shown are relative values that were normalized to β-actin or GAPDH, which quantify total genomic DNA. Standard curves were generated using a series of 8 dilutions of necropsy marrow DNA samples from each animal. PB, peripheral blood.

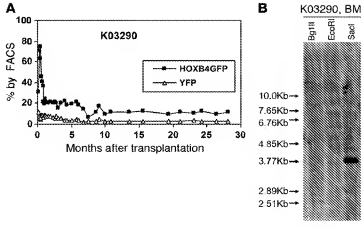


**Figure 5**
Characterization of macaque K03290, an animal with normal hematopoiesis. (**A**) Marking levels of K03290 in peripheral blood, which had been stabilized for more than 2 years. (**B**) Southern blot analysis showed multiple bands of HOXB4-overexpressing marrow cells. Digestion with SacI, which cut the transgene twice, showed a 3.9-kb band for all the integrants. BglII and EcoRI cut the transgene once, and so each band indicates an individual integrant.

*HOXB4 overexpression is responsible for leukemogenesis.* Flow cytometric analysis showed high-level expression of HOXB4GFP in these animals, which was confirmed by western blot (Figure 3A). To further explore the mechanisms underlying leukemogenesis, we established a leukemic cell line from G374 bone marrow; this line grows robustly in the presence of cytokines and expresses CD34. We designed shRNAs to *PRDM16*, *MYB*, and *HOXB4* to determine whether the increased expression of these genes has contributed to leukemogenesis. As shown in Figure 3B, down-regulation of *PRDM16* and *MYB* expression only decreased the cell proliferation rate during a 3-week culture period without a change in the percentage of $CD34^+$ cells and no differentiation. In contrast, downregulation of HOXB4 resulted in the complete disappearance of $CD34^+$ cells, and most cells expressed the mature granulocyte marker Dm5 after 2 weeks (Figure 3C). These findings demonstrate that *HOXB4* knockdown resulted in the differentiation of the leukemic cells. Furthermore, differentiated cells stopped growing after 3 weeks. These data demonstrate the ability of HOXB4 in concert with key cooperating genes to promote stem cell self-renewal and inhibit cell differentiation in leukemic clones, thus eventually leading to overt leukemia.

Taken together, these data suggest that HOXB4 played a pivotal role in leukemogenesis through its ability to effectively collaborate with cooperating mutagenic events.

*Dynamics of leukemic clones.* We used SYBR Green real-time PCR to track and quantitate the leukemic clones in the animals (Figure 4, A–C). Between 1 and 2 months after transplantation, only 0.01%–0.1% of peripheral blood cells were leukemic clones. By 6 to 12 months, the contributions increased to about 1% in peripheral blood and up to 10% in marrow cells. However, there was no significant change over the subsequent year. Interestingly, the leukemic clone in G374 marrow increased to 10% at 6 months after transplantation but decreased to less than 1% at 11 months after transplantation. These data are consistent with the flow cytometric results (Figure 1A and data not shown) and Southern blot analysis (Supplemental Figure 5).

The latency of about 1 year before the development of overt leukemia in all animals suggests that more mutations may have accumulated in that time period, leading to the development of leukemic blasts. Karyotype analysis of marrow cells from the 3 leukemic animals, however, did not detect any gross abnormalities (data not shown). This does not rule out the possibility of gene deletions and duplications and accumulation of random nucleotide mutations that would not have been detected by standard karyotype analysis.

*Characterization of another macaque with normal hematopoiesis.* One (K03290) of 2 macaques that have been followed for more than 28 months has not shown any evidence of abnormalities thus far. The marking levels in peripheral granulocytes have stabilized at 10% for HOXB4 and 2% for YFP (Figure 5A). Southern blot analysis of DNA from $HOXB4GFP^+$ marrow cells revealed multiple bands, indicative of polyclonal hematopoiesis (Figure 5B). LAM-PCR analysis of $HOXB4GFP^+$ cells showed 9 integration sites (Table 1), among which mitochondrial ribosomal protein S23 (*MRPS23*), *CD9*, *MMP8*, and *IGFBP8* have been reported to be involved in tumorigenesis. Thus, even with integration sites at oncogene loci, this macaque has not developed malignancy yet.

*No evidence of abnormalities in animals that received MGMT-transduced cells.* In contrast to the 3 of 4 animals that developed leukemia after transplantation of HOXB4-transduced cells, none of the more than 40 dogs and monkeys transplanted with transduced cells using transgenes other than HOXB4 and followed for an average of 3 years with marking levels greater than 1% have developed leukemia ($P < 0.001$). Among these animals were 5 dogs that received the MGMTGFP-transduced cells using the same murine stem cell virus–based (MSCV-based) retroviral vector as was used for the HOXB4 studies. These animals also received multiple rounds of chemotherapy after transplantation

**Table 1**
Integration sites retrieved from *HOXB4*-overexpressing cells in K03290

| Gene | Chromosome | Location |
|---|---|---|
| *MRPS23* exon reverse | 16 | 42076270 |
| *CD9* intron reverse | 11 | 6340380 |
| *MMP8* before TSS | 14 | 101345377 |
| *IGFBP7* before TSS | 5 | 72212336 |
| *MYH9* | 10 | 80333233 |
| *DYN* | 18 | 42102723 |
| *RUFTY2* | 2 | 135082832 |
| *LMNB1* | 6 | 123152116 |
| Predicted gene before TSS | 15 | 98031113 |

Several viral vector integration sites at known oncogene loci are identified.

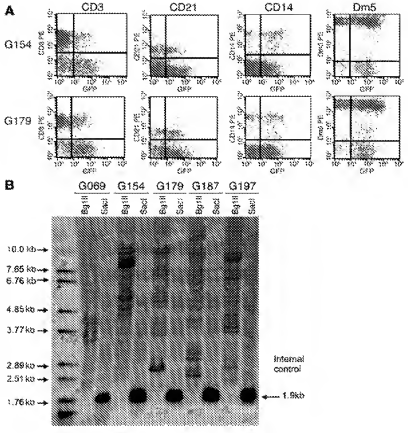


**Figure 6**
Normal hematopoiesis in dogs that received MGMT-transduced cells using MSCV retroviral vector. (**A**) Subset analysis shows marked cells in all the lineages of blood cells. Analysis of peripheral blood from 2 representative animals is presented. (**B**) Southern blot analysis of marrow from 5 animals demonstrates polyclonality. Digestion with BglII, which cuts vector once, releases a unique band for each integration sites, while digestion with SacI, which cuts vector twice, releases a single internal control band of 1.9 kb.

to increase the marking levels (25, 26). Five years after transplantation, the marking stabilized at high levels, in particular for granulocytes (Table 2). Peripheral blood and marrow analysis showed normal hematopoiesis with no evidence of leukemia (Figure 6A). Furthermore, Southern blot analysis of bone marrow DNA from these animals showed multiple bands, demonstrating polyclonality (Figure 6B).

Integration site analysis of the 5 animals and many other long-term follow-up animals has been published elsewhere (22, 27). Interestingly, we detected virus insertions at similar genes, including *CCDN3*, *JAK1*, *LMO2*, *MYH9*, and *PRDM16*, without the development of leukemia, further supporting the hypothesis that integrations at oncogene loci alone may not be sufficient to initiate leukemogenesis.

## Discussion

Here, we report that retrovirus-mediated HOXB4 expression in hematopoietic repopulating cells led to monoclonality and leukemia in 2 out of 2 dogs and 1 out of 2 macaques, approximately 2 years after transplantation. In contrast, more than 40 dogs and monkeys received retrovirus-modified CD34$^+$ cells with transgenes other than HOXB4, including 5 dogs that received MGMT-transduced cells using the same vector backbone as in the HOXB4 animals described here, and all of these animals have maintained normal polyclonal hematopoiesis after transplantation. A role of HOXB4 in the development of leukemia in these animals is further supported by HOXB4 knockdown experiments showing the differentiation of leukemic cells without HOXB4 overexpression.

Retroviruses have long been known to cause leukemia by insertional mutagenesis. Clonal dominance of hematopoietic stem cells by retroviral gene marking has been observed in a large group of mice (28, 29); furthermore, high-copy retroviral gene transfer of marker or drug resistance gene induced leukemia in mice due to combinatorial insertional mutagenesis (30, 31). Studies in nonhuman primates using retroviral vector preparations containing replication-competent viruses induced T cell lymphoma (32). Using replication-incompetent viruses, there has only been 1 report, by Seggewiss et al. (33), of insertional mutagenesis in nonhuman primates. The investigators found a fatal myeloid sarcoma in 1 monkey 5 years after transplantation with a MSCV-based RD114–pseudotyped retroviral vector. Interestingly this animal had repopulated with only 1 clone and had also received 1 cycle of chemotherapy. This animal was 1 of 7 animals that underwent transplantation using a similar vector and was followed for more than 5 years. These investigators also point out that this was the only animal that developed leukemia or abnormal hematopoiesis out of more than 80 large animals that have been followed long-term (33). These observations in large animals, together with clinical stem cell gene therapy trials for diseases other than SCID-X1, suggest that the development of leukemia induced by insertional mutagenesis alone occurs at very low incidence in large animals or humans, while gene therapy with a retroviral vector that expresses HOXB4 or IL2Rγ and possibly other growth-promoting genes significantly increases the incidence of leukemogenesis.

In our study, 3 of 4 animals that received HOXB4-overexpressing cells and had a follow-up of at least 2 years developed leu-

**Table 2**
Stable, high-level gene marking in granulocytes in dogs transplanted with MGMTGFP-transduced repopulating cells

| Animal | Follow-up (yr) | Marking in granulocytes (%) |
|---|---|---|
| G069 | 5.6 | 38 |
| G154 | 5.2 | 96 |
| G179 | 5.1 | 91 |
| G187 | 4.9 | 94 |
| G197 | 4.8 | 76 |
| Mean | 5.1 | 79 |

All animals received multiple cycles of chemotherapy for in vivo selection of gene-modified cells.

kemia, a higher incidence than in the SCID-X1 trials. This may be because HOXB4 promotes stem cell self-renewal and extensive expansion of myeloid progenitors (34), while IL2Rγ expands only lymphoid cells. All 4 cases in the SCID-X1 trials were T cell leukemias, while all 3 leukemias in our study were myeloid leukemias, which is unlikely to be random coincidence. We would assume that there is no difference between these viral vectors in the initial selection of integration sites. Instead, our data suggest that it is a consequence of oncogene collaboration (or integration site collaboration), as suggested in a mouse study in which cooperativity between LMO2 and IL2Rγ was documented (35). Studies in our other animals have identified integration sites at LMO2 (22). However, we did not detect any integrations in sites at LMO2 in the gene-modified cells of the animals reported here, although we can not rule out that this occurred in minor clones but at levels below detection and without apparent consequence, perhaps due to HOXB4's reported ability to moderately suppress lymphoid repopulation (36, 37). Based on the activation of nearby genes known to be involved in leukemogenesis, the integration sites observed in the leukemic clones are very likely a consequence of sustained in vivo proliferation and selection in HOXB4-transduced repopulating cells.

Both dogs and monkeys developed leukemia approximately 2 years after transplantation. The number of integrations associated with the canine leukemias (1 or 2 integrations) was lower than in the monkey (5 integrations). In our previous studies we observed that HOXB4 expression levels were higher in dog cells than in nonhuman primate cells and that HOXB4 overexpression allowed for immortalization of dog cells but not monkey cells (34), which would support a more pronounced effect of HOXB4 in dog cells. These findings suggest that dogs may be more susceptible to leukemic transformation by HOXB4 (and perhaps other growth-promoting genes) and retroviral insertional mutagenesis. This possibility further suggests that the canine model may prove more sensitive for preclinical safety testing of candidate retroviral gene therapy vectors.

Clone-tracking PCR analysis demonstrated a substantial clonal expansion during early engraftment followed by a dormant phase lasting about 1 year. Growing evidence suggests that oncogenes induce DNA hyperreplication and DNA damage and that unrepaired or misrepaired damage eventually leads to clones capable of uncontrolled expansion (38, 39). In addition, a cancer mutator strain theory proposes that clones with hypermutations are associated with tumorigenesis (40). Thus, we propose that oncogene activation may increase mutation rates and accumulation of malignant clones, eventually resulting in malignancies.

Interestingly, HOXB4 overexpression has not been associated with leukemia in mice, although several other wild-type HOXA9 (20, 41, 42) or mutant Hox fusions with NUP98 (42) are clearly leukemogenic. This may reflect differential potency of HOXB4 and other Hox transcription factors to have an impact on self-renewal versus differentiation in different species. We also speculate that the much larger cell dose used for transplantation in these large animal models and the attendant larger number of integration sites (e.g., here we transplanted some 1,000-fold more retroviral integration sites compared with the mouse studies) resulted in an increased chance of insertional mutagenesis. The increased time of follow-up — here, animals were followed for over a year, compared with the usually shorter times for mice — may also allow detection of long latency leukemias in larger animals. Together, these findings highlight the importance of using large animal models for preclinical safety testing of gene therapy approaches involving genetic manipulation of primitive hematopoietic cells.

The 5 animals that received MGMT-expressing cells showed normal hematopoiesis even at 5 years after transplantation and with similar integration sites at oncogene loci. Our observations emphasize that the benefits of stem cell gene therapy can outweigh its potential risks in cases like MGMT-based gene therapy, where transgene-expressing cells do not possess an intrinsic growth advantage over control cells.

In conclusion, our findings in 2 clinically relevant large animal models suggest that transgenes capable of expanding hematopoietic repopulating cells may pose an increased risk of leukemogenesis relative to transgenes that do not confer an intrinsic constitutive selective advantage. Thus, our data suggest the continued need for caution in genetic manipulation of repopulating cells, particularly when the transgene may confer an intrinsic selective growth advantage. In addition, large animal studies, in particular dog studies, should be highly valuable models in assessing safety of retroviral vectors.

## Methods

*Animals.* Nonhuman primates (*Macaca nemestrina*) were housed in the University of Washington National Primate Center, and dogs were housed at the Animal Health Resources unit of the Fred Hutchinson Cancer Research Center. All animal experiments and manipulations conducted were previously approved by the IACUCs of the Fred Hutchinson Cancer Research Center and the University of Washington.

*Transplantation of HOXB4-transduced cells.* Equal aliquots of MACS-purified dog $CD34^+$ cells were transduced with Phoenix RD114 pseudotyped gammaretroviral vector MSCV-HOXB4-ires-GFP or control MSCV-ires-YFP after 2 days of prestimulation in Iscove's modified Dulbecco's medium (IMDM) supplemented with 12.5% horse serum, 12.5% fetal bovine serum, $10^{-6}$ M hydrocortisone, $10^{-4}$ M β-mercaptoethanol, 2 mM glutamine, 1% penicillin/streptomycin (×100 liquid; Invitrogen) in the presence of Flt3-L, canine SCF, and canine G-CSF, each at 50 ng/ml. For transduction, cells were exposed to retroviral vectors at an MOI of 1–2 for 4 hours in the presence of growth factors. After overnight culture in media containing growth factors, cells were re-exposed to the same MOI of concentrated vector for 4 hours. Immediately after this second exposure, cells were washed and infused into lethally irradiated animal (920 cGy). The transduction and transplantation of macaque cells have been previously reported (21).

*FACS.* Flow cytometry was used to determine the marking levels of the animals after transplantation. Blood samples from control animals that do not express GFP were used for gating. For subset analysis of dog cells, CD3, CD21, CD14, CD34, and Dm5 antibodies were used. For subset analysis of macaque cells, CD3, CD20, CD14, CD13, and CD34 antibodies were used.

*Morphology.* Bone marrow biopsy or necropsy smears were prepared for morphological examination. Glass slides were stained with Wright-Giemsa stain. Digital pictures were taken with a Nikon microscope. Pathologists performed necropsy analysis of tissue samples from the dogs and monkey.

*Southern blot analysis.* Southern blot was performed to determine clonality. DNA from the marrow of animals was extracted using a Puregene DNA Purification Kit (Gentra Systems). DNA (20 μg) was digested overnight with BglII, EcoRI, or SacI and electrophoresed on 0.8% agarose gel. Denatured DNA was transferred to $N^+$ nylon membrane and hybridized in QuickHyb solution (Amersham) with $^{32}P$-labeled ires-GFP, which was cut from

the MSCV-HOXB4-ires-GFP plasmid. Hybridization signals were detected using a Typhoon Phosphor Imaging System (Amersham Biosciences).

*Integration site analysis.* Integration site analysis was performed using LAM-PCR as described previously (22). The PCR products were cloned into TOPO vector for sequencing. The retrieved sequences were aligned with dog genome assembly May 2005 or rhesus macaque genome assembly January 2006 at the University of California Santa Cruz (UCSC) Genome Browser Web site (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start&org=Dog [for dogs] and http://genome.ucsc.edu/cgi-bin/hgBlat?command=start&org=Rhesus [for rhesus]) to determine the integration sites.

*Knockdown experiments.* A leukemic cell line was derived from the marrow of dog G374 at necropsy. Cells were cultured in IMDM supplemented in 12.5% horse serum and 12.5% fetal bovine serum in the presence of canine SCF, thrombopoietin, and Flt3-L, each at 100 ng/ml. shRNA constructs were cloned into an LMP vector (Open Biosystems) that coexpressed puro$^r$ gene. Downregulation of the targeted genes was confirmed by real-time PCR. Phoenix RD114 pseudotyped viral vectors were produced by transient transfection of 293T cells. The leukemic cells were transduced with viral vectors and selected with 5 μg/ml puromycin the next day. Selected cells were kept in culture or harvested for RNA extraction and real-time RT-PCR analysis.

*Real-time RT-PCR.* Total RNA was extracted using RNeasy Mini Kit (Qiagen). Residual DNA was eliminated by DNase I treatment. Canine or macaque mRNA sequences were determined by aligning human RNA sequences with canine or macaque genome sequences at the UCSC Genome Browser Web site (http://genome.ucsc.edu/). Reverse transcription was performed using ThermoScript RT-PCR System (Invitrogen). Primers were designed with Primer 3.0 program. For quantitative analysis of gene expression, SYBR Green real-time PCR was performed on 7500 Real Time PCR System (Applied Biosystems). The data were normalized to GAPDH for monkey samples or to β-actin for dog samples.

*Clone tracking real-time PCR.* Two primers, one on MSCV transgene and another on genome sequence of the integration sites, were designed to track the contribution of the leukemic clone to hematopoiesis. For SYBR Green real-time PCR, genomic DNA samples from marrow at necropsy were used for generation of the standard curve, assuming that all the HOXB4GFP$^+$ cells were from the leukemic clone, which was supported by Southern blot data.

*Western blot.* Bone marrow cells from the leukemic animals and control normal animal were subjected to protein extraction and western blot analysis of HOXB4 expression, which has been detailed elsewhere (21).

*Statistics.* We analyzed the data by Fisher's exact test. *P* values less than 0.05 were considered to be statistically significant.

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Address correspondence to: Hans-Peter Kiem, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Mail Stop D1-100, PO Box 19024, Seattle, Washington 98109-1024, USA. Phone: (206) 667-4425; Fax: (206) 667-6124; E-mail: hkiem@fhcrc.org.

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# Differential and Common Leukemogenic Potentials of Multiple *NUP98-Hox* Fusion Proteins Alone or with Meis1

Nicolas Pineault,[1] Carolina Abramovich,[1] Hideaki Ohta,[1] and R. Keith Humphries[1,2]*

*Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver V5Z 1L3,[1] and Department of Medicine, University of British Columbia, Vancouver V5Z 4E3,[2] British Columbia, Canada*

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***NUP98-Hox* fusion genes are newly identified oncogenes isolated in myeloid leukemias. Intriguingly, only *Abd-B Hox* genes have been reported as fusion partners, indicating that they may have unique overlapping leukemogenic properties. To address this hypothesis, we engineered novel NUP98 fusions with Hox genes not previously identified as fusion partners: the *Abd-B*-like gene *HOXA10* and two *Antennepedia*-like genes, *HOXB3* and *HOXB4*. Notably, *NUP98-HOXA10* and *NUP98-HOXB3* but not *NUP98-HOXB4* induced leukemia in a murine transplant model, which is consistent with the reported leukemogenic potential ability of *HOXA10* and *HOXB3* but not *HOXB4*. Thus, the ability of *Hox* genes to induce leukemia as NUP98 fusion partners, although apparently redundant for *Abd-B*-like activity, is not restricted to this group, but rather is determined by the intrinsic leukemogenic potential of the Hox partner. We also show that the potent leukemogenic activity of *Abd-B*-like *Hox* genes is correlated with their strong ability to block hematopoietic differentiation. Conversely, coexpression of the Hox cofactor *Meis1* alleviated the requirement of a strong intrinsic Hox-transforming potential to induce leukemia. Our results support a model in which many if not all *Hox* genes can be leukemogenic and point to striking functional overlap not previously appreciated, presumably reflecting common regulated pathways.**



The clustered homeobox *Hox* genes encode a highly conserved family of transcription factors characterized by a 60-amino-acid DNA binding motif, the homeodomain. While *Hox* genes were first recognized for their prominent roles in embryonic development, their involvement in both normal and malignant hematopoietic processes is now well documented (2, 6, 30).

A central role for *Hox* genes in hematological malignancies is supported by the frequently observed elevation of *Hox* gene expression in acute myeloid leukemia (AML) (11, 14, 20) and the common involvement of their upstream regulator, *MLL*, in both myeloid and lymphoid leukemia (8). A more direct involvement for *Hox* genes in leukemia is supported by their frequent fusion to the nucleoporin gene *NUP98*. *Hox* genes are the most frequent fusion partner in an ever-growing number of *NUP98* fusion genes isolated almost exclusively in patients with myeloid leukemia (AML, posttherapy AML, and chronic myeloid leukemia). Reported Hox partners of *NUP98* include *HOXA9* (3, 21), *HOXD13* (27), *HOXA13* (9), *HOXA11* (9), *HOXC11* (35), and *HOXC13* (19). All NUP98-Hox fusions reported to date consist of the N terminus of NUP98, containing a region of multiple phenylalanine-glycine (FG) repeats that may act as a transcriptional coactivator through binding to CBP/p300 (13), and the C terminus of the *Hox* gene product, containing the intact homeodomain and various extents of flanking amino acids. The leukemogenic potential of such chimeric proteins was confirmed for *NUP98-HOXA9* (*NA9*) and *NUP98-HOXD13* (*ND13*) in retrovirally transduced murine bone marrow transplantation models with both genes inducing AML and/or a myeloproliferative syndrome (16, 25). The transforming potentials of NA9 and ND13 were found to be dependent on the ability of their homeodomains to bind DNA (13, 25), and the Pbx interacting motif of HOXA9 in NA9 was found to be dispensable, suggesting that the Hox DNA-binding domain is the minimal and possibly only significant contribution of the Hox fusion partner.

The leukemogenic activity of *Hox* genes is not restricted to chimeric NUP98-Hox proteins, since *HOXB8, HOXA10*, *HOXB3*, and *HOXA9* have previously been shown to induce AML upon engineered overexpression (15, 24, 31, 37). This suggests that a key result of NUP98 fusion with Hox is deregulated expression of Hox-responsive genes by the chimeric Hox protein, in which NUP98 replaces the transcriptional activity of the Hox N-terminal region (13). Consistent with this, both *HOXA9* and *NA9* studied in similar experimental settings are leukemogenic and block myeloid progenitor differentiation in interleukin-3 and granulocyte-macrophage colony-stimulating factor (7, 16). Nevertheless, differences in disease latency, cooperativity with Meis1, and other in vitro growth properties suggest that NUP98-Hox fusions have different properties compared to their normal counterparts. For instance, in vitro, *HOXA9* did not replicate the effect induced by *NA9*, such as proliferation in stem cell factor and granulocytic differentiation block with granulocyte colony-stimulating factor (7).

Intriguingly, so far only *Hox* genes belonging to the Abd-B-like Hox paralog groups (groups 9 to 13) have been characterized as *NUP98* fusion partners, and within those, only members from paralog groups 9, 11, and 13 have been observed. Thus, it may be that only a subset of *Hox* genes, sharing potential overlap in gene targets and/or pathways, can complement *NUP98*. Outside of the *Abd-B*-like *Hox* genes, it is of interest

* Corresponding author. Mailing address: Terry Fox Laboratory, 601 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada. Phone: (604) 877-6070, ext. 3095. Fax: (604) 877-0712. E-mail: khumphri@bccrc.ca.

that *HOXB4*, while having potent ability to enhance hematopoietic stem cell (HSC) expansion is on its own nonleukemogenic, whereas the near neighbor *HOXB3* can cause marked myeloproliferation and ultimately leukemia (1, 31). These findings raise the interesting question as to the intrinsic leukemogenic potential of *Hox* genes across the clusters and to their leukemogenic potential in the context of fusion genes.

The leukemic potential of intact *Hox* genes, such as *HOXB3* and *HOXA9*, and *NUP98* fusions with *HOXA9* or *HOXD13* has been shown to be increased by co-overexpression of *Meis1* (15, 22, 25, 36) as evidenced by decreased disease latency. The basis for this cooperativity is unclear because, although in vitro studies have suggested that *Meis1* expression can synergize with *Hox* genes in promoting a stronger differentiation block of Hox-transformed myeloid progenitors (5), it does not show leukemogenic activity on its own in vivo (16, 25). Understanding the role of *Meis1* is further complicated by the fact that several of the known collaborating Hox proteins involved lack the ability to physically interact with Meis1, as in the case of HOXB3 and ND13. Finally, it is not clear whether all NUP98-Hox fusions could be strongly complemented by *Meis1* since the leukemogenic potential of *NA9* was only weakly augmented by *Meis1*, whereas *Meis1* strongly complemented *ND13* (16, 25).

The principal objectives of the work described here were to test whether the preferential identification of *Abd-B*-like *Hox* genes as *NUP98* fusion partners in human leukemias is a consequence of their special intrinsic properties or, on the contrary, whether other *Hox* genes might share the functional capacity to form oncogenic *NUP98* fusion genes. The leukemogenic potential of various *NUP98-Hox* fusion genes representing *Hox* genes of different paralog groups and different leukemogenic potentials was tested in vivo, alone and in concert with *Meis1*, to enlarge the knowledge of the role of *Meis1* in Hox-related leukemia. Furthermore, in order to gain further insight into the mechanism(s) of Hox-mediated leukemia, we analyzed the impact of the native and *NUP98*-fused *Hox* genes on bone marrow differentiation and proliferation.

## MATERIALS AND METHODS

**Retroviral vectors and engineering of novel NUP98-Hox fusion genes.** The murine stem cell virus (MSCV) Flag-NUP98–HOXD13 (ND13) internal ribosomal entry site (IRES)-enhanced green fluorescent protein (eGFP), MSCV ND13 IRES-eGFP, MSCV Meis1 IRES-yellow fluorescent protein (YFP), MSCV HOXB4 IRES-eGFP, and MSCV HOXA10 IRES-eGFP viral vectors used were previously described (1, 4, 25). We engineered the fusion of the cDNA portion corresponding to the homeobox-containing exon of *HOXA10* (NUP98-HOXA10; NA10), *HOXB4* (NB4), and *HOXB3* (NB3) with the *NUP98* portion found in the *ND13* fusion gene, which consists of exons 1 to 12 of *NUP98* (Fig. 1A). All gene fragments were obtained by PCR from cloned cDNAs with the Platinum *Taq* DNA polymerase High Fidelity (Invitrogen, Burlington, Canada); the primer sequences are available in the appendix. Constructs were validated by sequencing and correct expression and transmission were confirmed by Western blot and Southern blot analysis (Fig. 1B and C).

**Mice and retroviral infection of primary bone marrow cells.** Parental strain mice were bred and maintained at the British Columbia Cancer Research Centre animal facility. Donors of primary bone marrow cells were (C57BL/6Ly-Pep3b × C3H/HeJ)$F_1$ mice and recipients were (C57BL/6J × C3H/HeJ)$F_1$ (B6C3) mice. Primary mouse bone marrow cells were transduced as previously described (25). Briefly, bone marrow cells were harvested from mice treated 4 days previously with 150 mg of 5-fluorouracil/kg (Faulding, Underdale, Australia) and stimulated for 48 h in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 10 ng of human interleukin-6 per ml, 6 ng of murine interleukin-3 per ml, and 100 ng of murine stem cell factor (StemCell Technologies

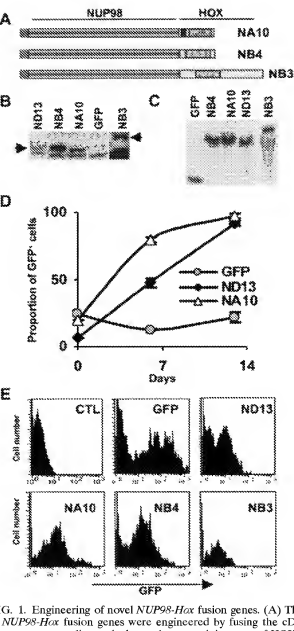


FIG. 1. Engineering of novel *NUP98-Hox* fusion genes. (A) Three new *NUP98-Hox* fusion genes were engineered by fusing the cDNA sequence corresponding to the homeobox-containing exon of *HOXA10* (NA10), *HOXB4* (NB4), and *HOXB3* (NB3) to that of *NUP98*. Only NA10 retained its Pbx-interacting motif, which is indicated as a black rectangle. The left grey boxes indicate the Flag tag, and the homeodomains are illustrated as light grey rectangles. (B) Western blot analysis of cell extracts from transduced BaF3 cells with a monoclonal Flag antibody. (C) Southern blot analysis showing full-length proviral integration of the various NUP98-Hox IRES-GFP viral vectors in transduced bone marrow cells. (D) *NA10* expression provides bone marrow cells with a growth advantage in vitro. The proportion of transduced $GFP^+$ bone marrow cells over time in liquid culture established with nonpurified transduced bone marrow cells is shown. Results of a representative experiment are shown: mean ± standard deviation of triplicate culture ($n = 3$). (E) Expression level of the GFP reporter gene in circulating leukocytes from mice transplanted with *NB4*, *NA10*, *NB3*, and *ND13* transduced bone marrow cells 8 to 13 weeks post-transplant. Histogram profiles of representative and GFP control (CTL) mice are shown.

TABLE 1. Engraftment levels in mice transplanted with singly transduced bone marrow cells 8 to 13 week posttransplant[a]

| Gene transduced | % GFP+ white blood cells ± SD | No. of blood cells | |
|---|---|---|---|
| | | White ($10^6$/ml) ± SD | Red ($10^9$/ml) ± SD |
| *GFP* (control) | 63.5 ± 11.5 (8)[b] | 7.9 ± 1.5 (6) | 10.8 ± 1.0 (7) |
| *NA10* | 70.7 ± 5.9 (7) | 7.7 ± 2.0 (7) | 11.3 ± 1.6 (3) |
| *NB4* | 69.5 ± 5.6 (10) | 7.8 ± 1.3 (10) | 9.8 ± 1.0 (9) |
| *A10* | 50.7 ± 14.5 (7) | 9.0 ± 3.6 (7) | 10.7 ± 1.1 (3) |
| *B4* | 63.6 ± 10.3 (6) | 6.9 ± 1.3 (6) | 9.1 ± 1.7 (6) |

[a] Results shown are means from at least two independent cohorts.
[b] Total number of recipients analyzed.

Inc., Vancouver, Canada) per ml. All bone marrow cultures used medium. The cells were infected by cocultivation with irradiated (4,000 cGy of X-ray) GP+ E-86 viral producer cells with the addition of 5 μg of protamine sulfate (Sigma, Oakville, Canada) per ml.

A 1:1 mixture of producers of the NUP98-Hox viruses (with the green fluorescent protein [GFP] marker) and Meis1 virus (with the YFP marker) was used to coinfect bone marrow cells. Loosely adherent and nonadherent cells were harvested from the cocultures after 2 days and cultured for 48 h in the same medium without protamine sulfate. Where indicated, transduced cells were highly purified based on expression of GFP, YFP, or both fluorescent proteins with a FACS-Vantage (Becton Dickinson, Mississauga, Canada), as previously described (25).

**Bone marrow transplantation and monitoring of recipients.** Purified GFP+ and/or nonpurified (unsorted cells) transduced bone marrow cells were injected into the tail vein of irradiated (900 cGy of $^{137}$Cs γ-radiation) recipient $F_1$ B6C3 mice. Mice transplanted with singly transduced bone marrow cells received from $1.4 \times 10^6$ to $3.5 \times 10^6$ GFP+ cells/transplant and mice injected with doubly transduced cells received from $0.2 \times 10^6$ to $8.0 \times 10^6$ GFP+/YFP+ bone marrow cells. For peripheral blood, bone marrow, and spleen cell analysis, single-cell suspensions were stained with the following monoclonal antibodies: phycoerythrin-labeled Gr-1, Mac-1, B220, Ter-119, and c-Kit (all obtained from Pharmingen, San Diego, Calif.). The immunoglobulin E (IgE) receptor was detected with a polyclonal biotinylated anti-IgE receptor antibody (StemCell Technologies Inc.) followed by streptavidin-phycoerythrin (Pharmingen, San Diego, Calif.). Morphological analysis of peripheral blood, bone marrow, and spleen cells and histological analysis were performed as previously described (25).

**In vitro assays.** Cell proliferation was assessed in the same media described above. Differentiation of clonogenic progenitors was analyzed by plating cells in 1 ml of methylcellulose culture medium per petri dish in standard conditions (Methocult M3434, StemCell Technologies Inc.), containing 10 ng of murine interleukin-3 per ml, 10 ng of human interleukin-6 per ml, 50 ng of murine stem cell factor per ml, and 3 U of human erythropoietin per ml. Colonies were scored microscopically with standard criteria after 8 to 10 days.

**CFU-S assay.** GFP+ purified bone marrow cells were first cultured for 7 days before injection. The total cell progeny from the starting equivalent (day 0) of 6 cells up to $1 \times 10^6$ cells were then injected into irradiated recipient mice. The recovery of CFU-spleen (CFU-S) cells was measured by determining the number of macroscopic colonies on the spleen at day 12 postinjection after fixation in Telleyesnickzky's solution.

**Southern blot analysis.** Genomic DNA was isolated with DNAzol reagent as recommended by the manufacturer (Invitrogen), and Southern blot analysis was performed as previously described (29). The stable integration of the provirus was confirmed by digesting the DNA with *Nhe*I (cutting in each long terminal repeat) and then the DNA-containing membrane was probed with $^{32}$P-labeled GFP cDNA. For clonality analysis DNA from NUP98-Hox plus Meis1 primary or secondary transplant recipients was digested with *Eco*RI and probed with a $^{32}$P-labeled NUP98 cDNA. DNA from *HOXB4*-plus-*Meis1*- and *HOXA10*-plus-*Meis1*-transplanted mice was digested with *Xba*I and *Eco*RI and hybridized to *HOXB4*- or *HOXA10*-specific probes, respectively.

**Western blot analysis.** Transduced BaF3 cells (infected with the same procedure as that for bone marrow) were lysed (400 mM NaCl, 0.1% Triton, 0.1 M HEPES, pH 7.5). Cell lysate was loaded onto a 10% Bis-Tris polyacrylamide gel (NuPAGE [NP0303], Invitrogen) and blotted to a Biotrace polyvinylidene difluoride membrane (Pall Corporation, Ann Arbor, Mich.). A monoclonal anti-Flag (M2, Sigma) and a donkey horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody were then used (Jackson ImmunoResearch Lab. Inc., West Grove, Pa.). Protein expression was detected with an enhanced Luminol reagent (Renaissance, Boston, Mass.).

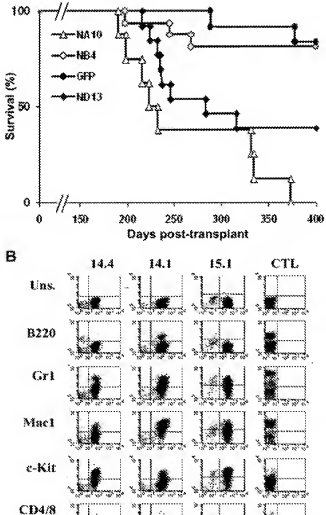


FIG. 2. Novel fusion gene *NUP98-HOXA10* but not *NUP98-HOXB4* induces leukemia in mice. (A) Survival curve of mice transplanted with *NA10* ($n = 8$), *NB4* ($n = 16$), *ND13* ($n = 13$), and GFP ($n = 12$) transduced bone marrow. (B) Immunophenotyping of leukemic cells. Flow cytometry profiles of bone marrow cells for three sick *NA10* recipients and one control mouse (CTL) are shown. Expression of GFP is shown on the *x* axis and that of the cell surface antigens on the *y* axis. Uns, unstained.

**Statistical analysis.** Data were statistically tested with the *t* test for dependent or independent samples (Microsoft Excel). Differences with *P* values <0.05 were considered statistically significant.

## RESULTS

**Redundancy of the ability of *Abd-B*-like *Hox* genes to cause AML as *NUP98* fusion partners.** The increasing list of *Abd-B*-like *Hox* genes that have been identified fused to *NUP98* in human AML strongly suggests a functionally equivalent role for this closely related group of genes in leukemia. Interest-

TABLE 2. Hematological characteristics of diseased *NA10* mice

| Mice (no.) | Mean day of sacrifice ± SD | Mean no. of white blood cells ($10^6$/ml) ± SD | Mean no. of red blood cells ($10^6$/ml) ± SD | Mean spleen wt (g) ± SD | Mean % blasts in marrow ± SD |
|---|---|---|---|---|---|
| *NA10* (5) | 257 ± 70.2 | 113.5 ± 149 | 2.7 ± 1.8 | 0.59 ± 0.27 | 36 ± 9 |
| Normal B6C3 control (3) | | 7.1 ± 6.6 | 6.8 ± 1.4 | 0.10 ± 0.02 | 8.2 ± 2.7 |

ingly, however, this list does not include any of the *Hox* genes belonging to the 10th paralog group, even though *HOXA10* has been reported to be leukemogenic in mouse models. This raises the possibility that *Hox* genes of the 10th paralog group do not possess the properties required to form leukemogenic *NUP98* fusion genes. To clarify this issue, we engineered a novel *NUP98-Abd-B* fusion gene, *NUP98-HOXA10* (*NA10*), and studied the effect of its overexpression in a mouse bone marrow transplantation model. To construct *NA10*, the cDNA portion corresponding to the second exon of *HOXA10*, containing its homeodomain and Pbx-interacting motif, was fused in frame to the *NUP98* portion found in *ND13*, thus recapitulating a typical *NUP98-Hox* fusion gene (Fig. 1). *NA10* cDNA was cloned into the MSCV-IRES-GFP retroviral vector, in which an internal ribosomal entry site allows the cotranslation of the GFP reporter gene and *NA10*.

Expression of *NA10* provided freshly transduced bone marrow cells with a strong growth advantage, as evidenced by the increasing proportion of transduced $GFP^+$ cells over time in liquid culture (Fig. 1D). This effect was similar to that induced by *ND13* and provided initial support for *HOXA10* as an oncogenic *NUP98* fusion partner. To determine whether the growth advantage could translate into leukemogenic activity in vivo, lethally irradiated mice were reconstituted with *NA10*-transduced bone marrow cells.

*NA10*-transduced bone marrow cells reconstituted the hematopoietic system of irradiated mice efficiently, with the proportion (Table 1) and expression levels of $GFP^+$ mononuclear cells (Fig. 1E) similar to that observed for ND13 control-transplanted mice. No significant differences were observed in the number of white blood cells and red blood cells in the *NA10* and control *HOXA10* and GFP recipients 8 weeks post-transplant (Table 1). However, as first detected at 24 weeks, three out of eight healthy-looking *NA10* mice displayed elevated white blood cell numbers ($14 \times 10^6$ to $35 \times 10^6$/ml) due to increased myeloid cells ($Gr1^+$ and/or $Mac1^+$), consistent with a mild myeloproliferative condition, as previously observed in some *ND13* and *NUP98-HOXA9* mice (16, 25).

Recipients transplanted with *NA10*-transduced bone marrow cells had a much reduced survival rate compared to GFP controls but similar to that observed for control *ND13* mice (Fig. 2A), with a median survival of 223 days. Examination of sick *NA10* recipients revealed that they had elevated white blood cell counts (3- to 50-fold above normal), anemia, and splenomegaly (Table 2). An AML phenotype was confirmed by Wright-Giemsa staining of bone marrow cytospins, which revealed a high proportion (≥25%) of blast cells in the marrow of the sick *NA10* recipients (Fig. 3A; Table 2). The myeloid nature of the leukemic cells was confirmed by flow cytometry (Fig. 2B), which revealed that the majority of the leukemic bone marrow cells stained positive for Mac1, Gr1, and c-Kit, although a small degree of phenotypic heterogeneity was observable among different mice. This phenotypic variability likely reflects small differences in the predominant stage of differentiation impairment influenced by the level of NA10 expression or the nature of an extra mutation(s) acquired during leukemia progression (see below).

All leukemias originated from transduced bone marrow cells, as confirmed by GFP expression (Fig. 2B) and Southern blot analysis, which detected the presence of the intact *NA10* provirus (data not shown). Histopathological analysis confirmed significant infiltration of immature myeloid cells in the spleen, liver, kidneys, and occasionally in lungs (data not shown). The leukemia was transplantable, and all secondary recipients injected with as few as 5,000 bone marrow cells also developed AML, with a latency ranging from 31 to 69 days (three primary mice tested). The much-reduced disease latency

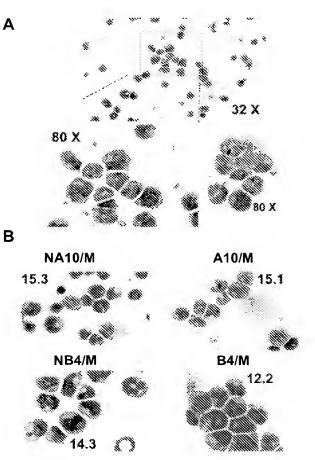


FIG. 3. Wright-Giemsa staining of leukemic bone marrow cells harvested from moribund *NUP98-HOXA10*, *NUP98-Hox/Meis1*, and *HOX/Meis1* mice. Staining of leukemic bone marrow cells harvested from moribund *NA10* mice (A) or from moribund *NUP98-Hox/Meis1* and *Hox/Meis1* mice (B). M, *Meis1*.

seen in the secondary transplants argues that additional mutations had been acquired during the genesis of the leukemias. As previously reported and confirmed here, control *HOXA10* (data not shown) and *ND13* recipients also developed AML over time (25, 37). In contrast to the reported disease kinetics observed between *HOXA9* and *NA9* mice (16), *HOXA10*, associated with a median survival of 151 days, induced AML faster than *NA10*. Together, these results demonstrate that the *Abd-B*-like *HOXA10* gene is a potent oncogenic *NUP98* fusion partner and support a model in which all members of this class of *Hox* genes have overlapping leukemogenic properties.

**Fusion of the nonleukemogenic *HOXB4* gene to *NUP98* does not cause AML.** The results presented above supported the hypothesis that *Abd-B*-like *Hox* genes have common transforming properties that can be complemented by fusion to *NUP98*. However, it remained possible that any *Hox* gene, regardless of its intrinsic leukemogenic potential, could form an oncogenic fusion gene with *NUP98*. To investigate this, we engineered a fusion between *NUP98* and *HOXB4*, a *Hox* gene previously found to be nonleukemogenic in transplant models and not yet implicated in human leukemia.

In contrast to *NA10*, *NB4* did not induce leukemia even after follow-up of 1 year (Fig. 2A). This lack of leukemogenic activity was not attributable to low-level engraftment or expression levels, as evident from GFP expression in peripheral blood mononuclear cells (Table 1 and Fig. 1E). During that observation period, three *NB4* and two *GFP* recipients died of unknown causes with no evidence of myeloproliferative disorder or hematological abnormalities. Clonogenic progenitor assays and fluorescence-activated cell sorter (FACS) analysis of the marrow of one of the *NB4* mice revealed no apparent perturbation, and all secondary bone marrow transplants remained healthy (>47 weeks). These results indicate that the fusion of *NUP98* to *HOXB4* was without detectable leukemogenic activity and thus suggest that not all *Hox* genes can form leukemogenic *NUP98* fusions.

To discriminate between the possibilities that *HOXB4* could not complement *NUP98* due to lack of intrinsic leukemogenic activity or that only *Abd-B* genes are able to collaborate with *NUP98*, we engineered a fusion between *NUP98* and another *Antennapedia*-like *Hox* gene, *HOXB3*, that on its own has been shown to induce AML. Low viral titers resulted in sufficient numbers of $GFP^+$ NB3 cells to transplant only two mice; nevertheless, one of two *NB3* recipients developed leukemia, as evidenced by a high white blood cell count ($110 \times 10^6$/ml), low red blood cell count ($5.3 \times 10^9$ red blood cells/ml), splenomegaly (0.58 g), and a proportion of blasts of 21% in its marrow at ≈235 days posttransplant, a latency similar to that reported for the intact *HOXB3* gene (31). Together, these data suggest that the ability of *Hox* genes to form leukemogenic fusions with *NUP98* is not restricted to *Abd-B*-like *Hox* genes, but may rather be determined by the intrinsic leukemogenic potential of the *Hox* gene fused.

**Redundant ability of *Hox* and *NUP98-Hox* genes to cause AML in collaboration with *Meis1* is independent of their intrinsic leukemogenic potential.** The Hox cofactor *Meis1* has been shown to reduce the time of disease onset for several *Hox*-induced leukemias, suggesting redundancy in the ability of *Hox* genes to collaborate with *Meis1*. A second important observation arising from these studies is that the strength of this cooperation seems to correlate with the intensity of the leukemogenic potential of the *Hox* gene involved. In order to clarify these issues, we investigated the potential synergy between *Meis1* and *NA10* (a strong oncogene), *NB3*, and *NB4* (nononcogenic). In addition, to determine whether the synergy between *Meis1* and the fusion genes reflects an intrinsic property inherited from their normal Hox counterparts, both *HOXA10* and *HOXB4* were coexpressed with *Meis1* in vivo.

Bone marrow cells were cotransduced with the various NUP98-Hox-IRES-GFP and Meis1-IRES-YFP retroviruses and injected into irradiated mice. Strikingly, all fusion and *Hox* genes tested collaborated with *Meis1* to induce leukemia, as evidenced by reduced disease latency or, in the case of *NB4* and *HOXB4*, conversion to leukemogenic activity (Fig. 4A and B). Expression of *Meis1* alone did not cause disease, as previously reported (15). Moreover, the median survival seen with *Meis1* in combination with *NA10* or *HOXA10* was much shorter than in combination with *NB3*, *HOXB4*, or *NB4* (Fig. 4B), reflecting the previously documented different leukemogenic potencies of the *Hox* genes by themselves. Moreover, although as single factors *NB4* and *HOXB4* were unable to induce leukemia, both genes were equally capable of inducing the disease with indistinguishable latency in combination with *Meis1* (Fig. 4A and B; Table 3). This ability of nonleukemogenic *Hox* genes to cause AML in collaboration with *Meis1* further supports the redundancy of such collaboration.

The phenotype of the diseased NA10/Meis1 (NA10/M), A10/M, NB4/M, and B4/M mice closely resembled that induced by *NA10* and that previously reported from ND13/M (25), with elevated white blood cell counts, anemia, splenomegaly, and a high percentage of poorly differentiated myeloid blasts in the bone marrow (Fig. 3B; Table 3). The myeloid phenotype of the leukemic cells was confirmed by flow cytometry (predominantly $Gr\text{-}1^+$ $Mac\text{-}1^+$ $B220^-$ $CD4/8^-$, data not shown). Importantly, flow cytometric data from representative mice (Fig. 5A) revealed that leukemic bone marrow cells were positive for coexpression of the transduced genes, as evidenced by the high proportion of GFP/YFP double-positive cells. The origin of the leukemias from cotransduced cells, initially a minority population (as low as 0.4%) in the transplant inocula, was further confirmed by Southern blot analysis, showing the presence of NUP98-Hox and Meis1 or Hox and Meis1 proviruses in primary and secondary leukemic mice (Fig. 5B).

The leukemias caused by NA10/M, A10/M, NB4/M, and B4/M were transplantable, with secondary transplant recipients succumbing to leukemia in 20 to 70 days posttransplant. The frequency of the leukemic initiating cell was estimated by injecting different doses of primary leukemic marrow cells into multiple recipients. All mice from all gene combinations died after receiving as few as 5,000 cells, and the majority of mice receiving 500 cells also succumbed to AML (Table 4), indicating a high frequency of leukemia-initiating cells. Clonal analysis of genomic DNA harvested from the primary and secondary transplants revealed that the majority of the leukemias were of clonal and/or oligoclonal origin, as evidenced by the same banding pattern of equal intensity in the primary and secondary transplants (Fig. 5C). Interestingly, some sick NB4/M and B4/M mice showed a polyclonal bone marrow reconstitution at the time of analysis, as evidenced by the presence of multiple NB4 or B4 proviral bands in the primary

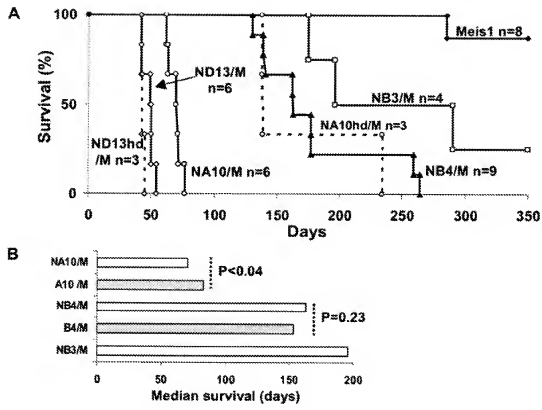


FIG. 4. Coexpression of *Meis1* strongly increases the leukemogenic potential of *NA10* and induces AML with *NB4*, *NB3*, and two native *Hox* genes. (A) Survival curve of mice transplanted with unsorted or enriched cotransduced bone marrow cells (see Materials and Methods for details). (B) The median disease latency for *HOXA10* plus *Meis1* (M) and *NA10* plus *Meis1* is shorter than that for *HOXB4* plus *Meis1*, *NB4* plus *Meis1*, or *NB3* plus *Meis1*. The *t* test *P* values are also shown.

recipients (see NB4/M 10.1 and 15.1 and B4/M 12.1 lanes in Fig. 5C), which is consistent with *HOXB4*'s intrinsic property of enhancing HSC regeneration capacity, a property that seems preserved in *NB4*.

Previous studies of *ND13* revealed that its growth-promoting activity was dependent on an intact homeodomain. These results suggested that the homeodomain might possibly be the most significant contribution of the Hox partner. To determine whether *Meis1* can collaborate with *NUP98* fusion genes containing only the homeodomain encoded by the *Hox* gene, a series of mice were transplanted with bone marrow cells cotransduced with *Meis1* and *ND13hd* or *NA10hd*, in which only the homeodomain (hd) encoded by *HOXD13* (amino acids 265 to 325) or *HOXA10* (amino acids 324 to 385) was fused in frame to *NUP98*. As seen in Fig. 4A, *ND13hd* was as efficient as *ND13* in inducing leukemia in collaboration with *Meis1*, and though following a longer latency, all mice transplanted with NA10hd/Meis1-transduced cells also succumbed to leukemia, indicating that the Hox homeodomain alone, for these genes, is enough for collaboration with *Meis1*.

**Strong leukemogenic potential of *Abd-B*-like *Hox* genes correlates with their robust ability to promote cell growth and block differentiation.** Based on the results described above, we speculated that the high leukemogenic potential of *Abd-B*-like *Hox* genes might be explained by their strong ability to promote cell proliferation and/or block differentiation.

All three novel fusion genes enhanced the proliferation of transduced bone marrow cells in short-term liquid culture, as

TABLE 3. Hematological characteristics of moribund mice transplanted with bone marrow cells cotransduced with *Meis1* and *Hox* or *NUP98-Hox* genes

| Genes transduced (no. of mice) | Mean time of sacrifice (days posttransplant) ± SD | Mean no. of blood cells ± SD | | Mean spleen wt (g) ± SD | % Bone marrow cells ± SD | |
|---|---|---|---|---|---|---|
| | | White ($10^9$/ml) | Red ($10^9$/ml) | | Blasts | Myeloblasts |
| *A10/M* (3) | 106.7 ± 27.7 | 115.8 ± 89.4 | 1.95 ± 1.0 | 0.56 ± 0.16 | 23.3 ± 5.7 | 55.7 ± 2.5 |
| *NA10/M* (3) | 69.7 ± 7.5 | 140 ± 133 | 5.5 ± 0.9 | 0.41 ± 0.11 | 32.0 ± 8.9 | 50.7 ± 4.7 |
| *B4/M* (4) | 144.3 ± 9.2 | 131.3 ± 63.7 | 4.0 ± 2.4 | 1.0 ± 0.5 | 28.0 ± 9.1 | 53.8 ± 3.8 |
| *NB4/M* (4) | 167.8 ± 21.0 | 65.5 ± 45.0 | 5.1 ± 2.9 | 0.7 ± 0.4 | 20.0 ± 6.0 | 46.0 ± 8.5 |
| Normal B6C3 (3) | | 7.1 ± 6.6 | 6.8 ± 1.4 | 0.10 ± 0.02 | 8.2 ± 2.7 | 10.4 ± 4.1 |

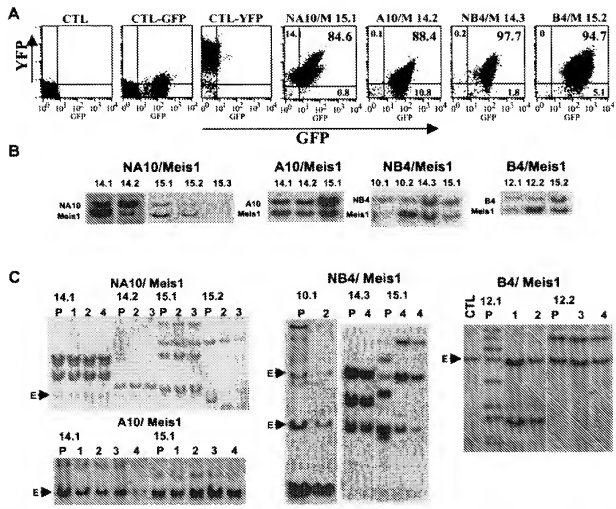


FIG. 5. *NUP98-Hox/Meis1-* and *Hox/Meis1*-induced myeloid leukemias are of clonal or oligoclonal origin. (A) Analysis of GFP and YFP expression in leukemic bone marrow cells (representative profiles shown). Fresh normal marrow (control, CTL) and GFP- and YFP-transduced control bone marrow cells are also shown. (B) Southern blot analysis from genomic DNA harvested from the leukemic marrow confirmed the presence of the Meis1-IRES-YFP and NUP98-Hox-IRES-GFP proviruses in the diseased mice. (C) To estimate the frequency of leukemia-initiating cells and analyze the clonal origin of the leukemias, bone marrow from moribund primary recipients was injected into irradiated secondary recipients at various doses. The lanes loaded with the primary recipient genomic DNA are indicated by P, and the lanes indicated by the numbers 1, 2, 3, and 4 correspond to DNAs of the secondary transplant recipients which received $5 \times 10^5$, $5 \times 10^4$, $5 \times 10^3$, and 500 bone marrow cells, respectively, from the primary recipients. The arrows indicate the locations of endogenous *Hox* or *Meis1* sequences hybridizing to the probes.

evidenced by the increased proportion of $GFP^+$ cells over time (Fig. 1D and data not shown). The total expansion of $GFP^+$ cells (i.e., total number of $GFP^+$ cells after 14 days/input $GFP^+$ cells) induced by *NA10* was, however, consistently greater (10- to 50-fold higher) than that induced by *NB3* and *NB4* and similar to that obtained for *ND13* (Fig. 6A). Moreover, this differential growth-promoting effect induced by the various fusion genes was also seen in more primitive cells, as shown by day 12 CFU-S. As previously reported, GFP cultures yielded a limited number of day 12 CFU-S cells following 1 week of culture. In contrast, all fusion genes tested produced a prominent increase, an average of 4,700-fold, in the yield of CFU-S-like cells (Fig. 6B). Again, the magnitude of the impact was greater for *NA10*, which produced the highest day 12 CFU-S recovery, matching that observed with *ND13* (Fig. 6B), whereas *NB4* consistently produced the smallest yield in all experiments ($n = 3$), followed by *NB3*.

The impact on bone marrow differentiation was further investigated following a culture period of 3 to 4 weeks. The majority of cells in the $GFP^+$ control cultures had undergone terminal differentiation, as evidenced by their slow growth and morphology (Fig. 7A; increased cytoplasm with increased granular content). FACS analysis (Fig. 7B) indicated a high level of expression of the c-Kit and IgE receptors, consistent with a mast cell phenotype. Under the same conditions, cultures initiated with *NB4*- and *NB3*-transduced cells consisted of a mixed population of well-differentiated and more primitive myeloid cells (Fig. 7A and B). Most strikingly, *NA10*- and *ND13*-transduced cells yielded cells almost entirely of an immature phenotype, as shown by negative expression of IgE receptors and low levels of c-Kit (Fig. 7B), blast morphology (Fig. 7A), and high proliferation rate. The greater impact on bone marrow differentiation observed for *NA10* was also evidenced in vivo. Phenotypic analysis of peripheral blood of

TABLE 4. Leukemias induced by *NUP98-HOX* fusion genes plus *Meis1* are highly transplantable

| Gene combination (no. of mice tested) | % of mice surviving 125 days after transplantation with indicated no. of leukemic bone marrow cells: | | Median survival (days) of mice receiving 5,000 leukemic cells |
|---|---|---|---|
| | 500 | 5,000 | |
| *NA10/Meis1* (4) | 33 | 0 | 19.5 |
| *A10/Meis1* (4) | 25 | 0 | 45 |
| *ND13/Meis1* (3) | 0 | 0 | 27 |
| *NB4/Meis1* (4) | 25 | 0 | 46 |
| *B4/Meis1* (4) | 50 | 0 | 69 |

*NA10*- and *HOXA10*-transplanted mice before the onset of evident disease also showed evidence of differentiation block, with a significant ($P < 0.03$) decrease of circulating $B220^+$ $GFP^+$ B cells (Fig. 7C). In contrast, a normal distribution of lymphoid and myeloid $GFP^+$ cells was observed in *NB4* and *HOXB4* recipients (Fig. 7C).

The different transforming potential and the block in bone marrow differentiation induced by the fusion genes were further confirmed by serial replating of bone marrow clonogenic progenitor cells in methylcellulose. Control $GFP^+$ cells only produced small clusters of myeloid cells (averaging $1.23 \times 10^3 \pm 5.6 \times 10^2$ cells/colony) in secondary plating, whereas all *NUP98-Hox* genes produced larger secondary granulocyte-macrophage myeloid colonies (ranging from $36 \times 10^3$ to $100 \times 10^3$ cells/colony). Consistent with the stronger differentiation block induced by *NA10* and *ND13*, bone marrow transduced with these genes formed large granulocyte-macrophage colonies over at least four passages (Fig. 7D). In contrast, *NB4*- or *NB3*-transduced clonogenic progenitors were essentially exhausted by the third replating (Fig. 7D; $n \geq 2$).

## DISCUSSION

In this study, we explored whether *Hox* genes have unique or overlapping or redundant roles in leukemogenesis in the context of *NUP98-HOX* fusion genes. Specifically, with novel *NUP98-HOX* fusion gene constructs, we addressed whether *Abd-B*-like *Hox* genes have common intrinsic properties that could make them prime *NUP98* fusion partner candidates, or whether the required properties could extend to other *Hox* members that would suggest a wider overlap in Hox function. Although the engineered *NUP98-Hox* genes originated from two different clusters and four different paralog groups, all four fusion genes tested produced a significant overlap in effects when expressed in bone marrow cells in vitro, reminiscent of the functional redundancy commonly observed for the *Hox* gene family. However, not all *Hox* genes formed strong leukemogenic *NUP98* fusion genes, but under certain circumstances, such as increased *Meis1* expression, all induced myeloid leukemia.

Decisively, the *Abd-B*-like *Hox* genes formed NUP98 chimeric proteins with the greatest impact on proliferation and differentiation in vitro and leukemogenic potency in vivo, which may account for the fact that to date, only *Hox* genes belonging to this group have been found rearranged with

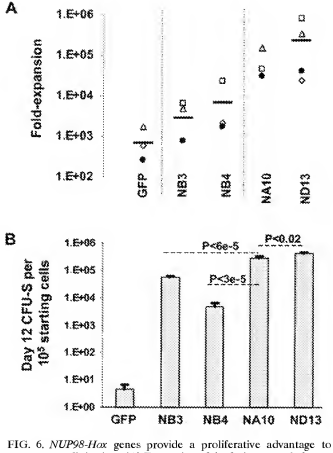


FIG. 6. *NUP98-Hox* genes provide a proliferative advantage to bone marrow cells in vitro. (A) Expression of the fusion genes led to an increased expansion of the transduced bone marrow cells compared to that of $GFP^+$ control or untransduced cells. Expansion for transduced bone marrow cells in liquid culture over 14 days and the mean (horizontal bar) of at least three experiments are shown. Expansion of cells in *HOXB4*- or *HOXA10*-transduced cells in culture ranged from $2.1 \times 10^5$ to $3.6 \times 10^5$ and $1.0 \times 10^5$ to $3.1 \times 10^5$, respectively. (B) Greater yield of day 12 CFU-S colonies after 1 week of culture of $10^5$ bone marrow cells transduced with fusions of *NUP98* and *Abd-B*-like *Hox* versus *NB3* and *NB4* (representative experiment shown ± standard deviation [$n = 3$]). The *P* values from the *t* test are also shown ($n = 3$).

*NUP98* in human leukemia. Nevertheless, the leukemogenic potentials observed for *NUP98-HOXB4* and *HOXB4* with *Meis1* support a model in which many if not all *Hox* genes may have the not previously appreciated capacity to contribute to leukemic transformation and point to striking overlap in properties.

**Intrinsic leukemogenic potential of intact *Hox* genes correlates with their ability to induce AML as *NUP98* fusion partners.** The novel *NA10* fusion gene induced AML with kinetics similar to that of *ND13*, indicating that the reported leukemogenic potential of *HOXA10* (37) is retained in the *NA10* fusion. Our data further imply that the ability to form leukemogenic *NUP98-Hox* fusion genes is not restricted to the already identified *Hox* genes, but rather seems to be a redundant property of *Abd-B*-like *Hox* genes. These results are consistent with reported functional identity between paralogous as well as nonparalogous *Abd-B*-related *Hox* genes (10, 38, 39). *Abd-B*-like *Hox* genes are derived from a common ancestor, and

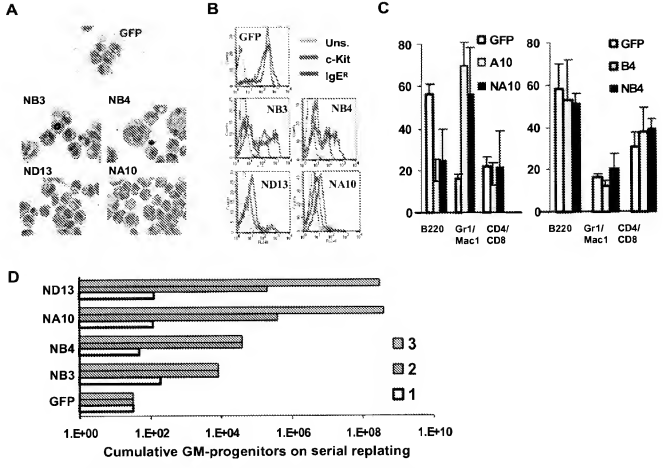


FIG. 7. *NUP98–Abd-B*-like *Hox* fusion genes have a greater transforming potential in vitro. (A) Wright-Giemsa staining of transduced bone marrow cells grown for 3 weeks in liquid culture. Magnification, ×80. Note the reduced number of mature granulocytic cells for *NA10*- and *ND13*-transduced cells compared to more differentiated $GFP^+$ control cells (representative experiment shown [$n = 2$]). (B) Immunophenotyping of cells after liquid culture. Unstained cells are in grey; IgE receptor ($IgE^R$) and c-Kit are shown in blue and red, respectively. (C) Lineage distribution of $GFP^+$ cells in transplanted mice. Mean distribution for the cohorts ± standard deviation. Analysis was done 24 weeks posttransplant for *GFP* ($n = 3$), *A10* ($n = 2$), and *NA10* ($n = 4$) mice and 30 weeks posttransplant for *B4* ($n = 3$) and *NB4* ($n = 3$). (D) Total number of granulocyte-monocyte progenitors in serial replating. Cumulative yield is shown for the initial plating of 750 transduced bone marrow cells (representative experiment shown [$n \geq 2$]).

therefore their homeodomains are closely related, which may indicate that their redundant function in leukemogenesis is mediated through the regulation of a similar or overlapping set of genes and/or pathways that control hematopoietic cell growth and differentiation.

Our results showing strong leukemogenic potential for *NA10* but not for *NB4* suggest that the intrinsic leukemogenicity of *NUP98* fusions is determined primarily by the *Hox* fusion partner. Moreover, such intrinsic leukemogenicity appears not to be restricted to *Abd-B*-like *Hox* genes, as suggested by leukemia arising in an *NB3* recipient. This differential ability of *NB3* and *NB4* to cause leukemia is consistent with the specific effects of their native counterparts (30, 31) and further demonstrates that closely related *Hox* genes are not completely functionally equivalent (26, 40).

AML is characterized by the uncontrolled proliferation of myeloid cells that accumulate at different stages where their further differentiation is blocked. The high correlation observed between the leukemogenic potential of the native and *NUP98-Hox* genes and their ability to increase self-renewal and block differentiation argues that the latter characteristics determine the Hox leukemogenic potential. Thus, the intrinsic ability of *ND13* and *NA10* and that reported for *NA9* (7) to sustain increased self-renewal and strongly block bone marrow differentiation appear to be a unifying property that determines their potent leukemogenicity.

**Redundant ability of *Hox* genes to collaborate with *Meis1* to induce AML.** Despite considerable differences in the function and structure of the native and *Hox*-fused genes studied in this work, they were all able, though with different latencies, to cause AML in collaboration with *Meis1*. Importantly, our data demonstrating the capacity of *NB4* and *B4* to cause AML with *Meis1* indicate that even *Hox* genes that do not cause leukemia on their own possess the redundant ability of becoming leukemogenic in the presence of this, and possibly other, cofactors.

Work from Calvo et al. showing that *Meis1* may further block differentiation of myeloid progenitors overexpressing *HOXA9* (5), coupled to the ability of *HOXB4* to transform fibroblasts in vitro (17), could in part explain the leukemogenic activity of *HOXB4* and *NB4* in collaboration with *Meis1*. Meis1

has been shown in myeloid cells to be associated with ternary complexes containing Pbx and Hox proteins (34), in which Meis1 directly interacts with Hox proteins from paralog groups 9 to 13, or indirectly associates with paralogs 1 to 8 through Pbx (33). NA10 and NA9 both retain the Pbx interacting motifs and therefore, Meis1 may indirectly interact with these proteins through the formation of trimer complexes containing Pbx, as suggested for A9-mediated transformation of myeloid progenitors (32). Arguing against the likelihood that such direct interactions are the key to leukemic transformation, several of the fusions tested (NB3, NB4, ND13, ND13hd, and NA10hd) lacked both known Pbx1- and Meis1-interacting motifs. Nevertheless, the longer latency observed for NA10hd/Meis1 (lacking a Pbx-interacting motif) compared to NA10/Meis1 (containing a Pbx-interacting motif) suggests that interaction with Pbx proteins, though dispensable, may accelerate disease development.

How *Meis1* accelerates the onset of Hox-mediated AML is still unclear. Whether *Meis1* functions to increase the strength of the transcriptional activity of *Hox* genes on Hox-regulated promoters or works through different target genes needs to be resolved. Several non-mutually exclusive explanations could be proposed. First, in view of work from Saleh et al. showing that under some circumstances Pbx1 can repress *Hox* gene-mediated transcription by interacting with multiple histone deacetylases (28) and a recent report showing increased ability of *HOXB4* to enhance self-renewal of HSC when Pbx1 was down-regulated (18), one could propose that Meis1, known to form strong heterodimers with Pbx1, might bind and titrate off Pbx proteins. Another possibility is that the exogenous Meis1 physically interacts with endogenously expressed Hox proteins also implicated in leukemia, such as Hoxa7 and Hoxa9 (7, 25). *Meis1* might also directly bind endogenous Hox-responsive genes and/or different genes, through Hox-independent activity (23). This possibility is supported by data indicating that Homothorax, a *Drosophila* Meis1 orthologue, possesses transcriptional activation activity of its own (12).

The *NUP98-Hox* fusion gene transformation models exploited here should now prove useful to dissect the molecular and cellular pathways involved in the redundant and differential leukemogenic potential of *Hox* genes and to provide new strategies to elucidate the role of collaborating genes.

## APPENDIX

**Supplementary materials.** Primer sequences to amplify indicated Hox regions for NUP98-HOX fusion constructs were as follows: NUP98 portion, forward, 5-AGTCGGATCCTTTAACAAATCATTTGGAACACCCTT-3, and reverse, 5-TTTAAGAATTCTACTGGGGCCTGGGG-3; HOXB4 Exon2, forward, 5-CCCAGGAATTCGTAAACCCCAATTACGCC-3, and reverse, 5-ACATAAAAATGCGGCCGCTAGAGCGCGCGCGGGGCC-3; HOXB3 Exon4, forward, 5-ATACCCGAATTCGCAGAGGGCTGTGGT-3, and reverse, 5-CCCATCCCTAATCCTCGAGCGC-3; HOXA10 Exon2, forward, 5-TTATCCGAATTCGGCAATTCCAAAGGTGAA-3, and reverse, 5-TTCAATAATGCGGCCGCTCATCAGGAAAAATT-3; HOXA10 homeod omain, homeobox (hd) A10hd; forward, 5-ACTGATGAATTCAAGAGTGGTCGGAAGAAGCGC-3; reverse, 5-ATAAAAATGCGGCCGCTTCACATTTTCTTCAGTTT-3; HOXD13 homeodomain, forward, 5-ACTGATGAATTCCGAAGAGGAGGAAGAAG-3, and reverse, 5-ACTGATCTCGAGTCATTTCTTGTCCTTCACTCTTCGGT-3.

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# Side effects of retroviral gene transfer into hematopoietic stem cells

Christopher Baum, Jochen Düllmann, Zhixiong Li, Boris Fehse, Johann Meyer, David A. Williams, and Christof von Kalle

**Recent conceptual and technical improvements have resulted in clinically meaningful levels of gene transfer into repopulating hematopoietic stem cells. At the same time, evidence is accumulating that gene therapy may induce several kinds of unexpected side effects, based on preclinical and clinical data. To assess the therapeutic potential of genetic interventions in hematopoietic cells, it will be important to derive a classification of side effects, to obtain insights into their underlying mechanisms, and to use rigorous statistical approaches in comparing data. We here review side effects related to target cell manipulation; vector production; transgene insertion and expression; selection procedures for transgenic cells; and immune surveillance. We also address some inherent differences between hematopoiesis in the most commonly used animal model, the laboratory mouse, and in humans. It is our intention to emphasize the need for a critical and hypothesis-driven analysis of "transgene toxicology," in order to improve safety, efficiency, and prognosis for the yet small but expanding group of patients that could benefit from gene therapy. (Blood. 2003; 101:2099-2114)**



## Effects and side effects in hematopoietic gene therapy

"This is a strange drop in my blood" (Goethe).

"It is the dose that makes the poison" (Paracelsus).

"What can go wrong, will go wrong" (Murphy).

Hematopoietic stem cells (HSCs) are important targets for somatic gene therapy, considering their availability for in vitro manipulation and their enormous biologic capacity.[1,2] In selected entities, gene therapy involving manipulation of HSCs has now clearly shown clinical efficiency, opening up new perspectives for the entire field.[3,4] However, it is a principle in pharmacology that no true effect is possible without inducing side effects. Prognosticating the type and incidence of side effects is an important step toward predicting the overall therapeutic benefit for a new modality.

The genetic modification of HSCs generates special concerns:

1. These cells are long-lived and might represent a reservoir for the accumulation of proto-oncogenic lesions.[5]

2. Current technology requires that HSCs have to be enriched and cultured in vitro to become accessible to genetic manipulation.

3. This also implies that the engineered graft represents only a small fraction (probably about 1%-10%) of the hematopoietic cell pool of a healthy individual. Infused cells may therefore be altered not only in terms of quality, but will also be heavily diluted by unmodified counterparts residing in the body. This may result in the establishment of a "strange drop in the blood," which could correct diseases only if it were strongly enriched in vivo.

4. Therefore, achieving targeted amplification or preferential survival of engineered cells is one important key to success in hematopoietic gene therapy.[2-4] However, clonal expansion, while limited by cellular senescence and exhaustion,[6] has also been suggested as a risk factor contributing to cellular transformation, at least when occurring under nonphysiologic conditions of growth.[7]

5. HSCs, or at least the cell preparations enriched for HSCs, may not only reconstitute the entire myeloerythroid and lymphoid spectrum, but they may also differentiate into or fuse with other cell types, including endothelial; skeletal and heart muscle cells; hepatocytes; neurons; and epithelial of gut and lungs. However, the frequency of such events is controversial.[8-12] The developmental potential of HSCs generates a huge repertoire of conceivable biologic conditions and anatomic sites where side effects may manifest. However, the likelihood of manifestations outside the hematopoietic system appears to be relatively low unless special triggers exist that drive fate-switching.[11,12]

6. Because of the high proliferative potential of HSCs, stable, heritable gene transfer is required for successful genetic modification. In the current "state-of-the-art" only viral vectors on the basis of retroviruses (including lentiviruses) mediate a predictable efficiency of stable transgene insertion with a predefined copy number.[13] Chromosomal insertion guarantees transgene maintenance during clonal amplification. Episomally persisting viral vector systems such as those based on Epstein-Barr virus are still suboptimal[14] because efficient gene transfer into HSCs is either not yet available or maintenance and expression of transgene copies are insufficiently investigated. Physicochemical methods result in a low probability for stable transgene insertion ($< 10^{-4}$).[15] Their efficiency may be increased when combined with endonucleases from retrotransposons or site-specific integrases.[16] Adeno-associated viruses (AAVs) also have a low and variable rate of stable insertion.[13] Recent advances in adenoviral vector technology may

From the Department of Hematology and Oncology, Hannover Medical School, Hannover, Germany; the Division of Experimental Hematology, Cincinnati Children's Research Foundation, Cincinnati, OH; the Departments of Neuroanatomy and Bone Marrow Transplantation, University Hospital Eppendorf, Hamburg, Germany; and the Department of Cell and Virus Genetics, Heinrich Pette Institute, Hamburg, Germany.

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Reprints: Christopher Baum, Experimental Cell Therapy, Department of Hematology and Oncology, Hannover Medical School, Carl-Neuberg-Straße 1, D-30625 Hannover, Germany; e-mail: baum.christopher@mh-hannover.de.

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**Table 1. Categories of side effects in the genetic manipulation of hematopoietic stem cells**

| Level | Category | Example |
|---|---|---|
| 1 | Culture | Loss of homing potential |
| 2 | Vector | Fusiogenic properties of viral envelope proteins |
| 3 | Genotoxicity | Insertional mutagenesis |
| 4 | Phenotoxicity | Interference of transgene product with cellular signaling networks |
| 5 | Selection toxicity | Side effects of regimens used to selectively engraft or expand manipulated cells |
| 6 | Immune response | Elimination of modified transgenic cells by antibodies or cytotoxic T cells directed against transgene-encoded antigens |
| 7 | Interactions of 1-6 | Cooperation of 3, 4, and 5 |

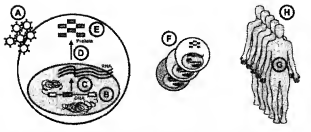


**Figure 2. Overview of dose issues in stem cell gene therapy.** The following items are indicated in the lettered circles: A, particle dosage; B, number of inserted genes; C, transcription; D, RNA processing; E, protein features; F, target pool size; G, cellular life span and plasticity; and H, number of patients.

increase their potential for stable gene delivery.[17] However, the utility of all of these alternative methods for transduction of HSCs with a defined and persisting transgene copy number is still unknown, as is the genetic risk associated with transgene insertion through these modalities.

7. The use of retroviral (including lentiviral) vectors implies that engineered cells of the same graft will vary with respect to transgene insertion sites (which are unpredictable and can affect both transgene and cellular gene expression), copy number per cell (which can be controlled more easily, but not entirely), and sequence (which can be modified in the error-prone process of reverse transcription). This produces a mixed chimerism of genetic modification in different stem cell clones, each with a theoretically distinct potential for eliciting side effects.

To facilitate the evaluation and discussion of side effects, we introduce a classification system at this point (Table 1).

As the whole process of genetic manipulation of transplantable HSCs is complex (Figure 1), problems may be encountered at different levels: (1) enrichment and culture of target cells (toxicity of cell manipulation); (2) vector production (vector toxicity); (3) insertion of foreign sequences or other alterations of the cellular genome (genotoxicity); (4) expression of transgenes (for which we would like to introduce the term "phenotoxicity"); (5) conditioning or selective drugs for enrichment of gene-manipulated cells (selection toxicity); (6) immune responses evoked by vector components or the transgene product (immunogenicity); and (7) aggravating interactions of some of these events.

Depending on the type, severity, and kinetics of side effects, patients may be asymptomatic or present with unclear symptoms, such as fever of unknown origin, signs of hemolysis, cytopenia of any lineage, immunodeficiency, autoimmune disorders, myelodysplasia, or, at worst, lymphoma, leukemia, or other types of malignancy. Some of these disorders, most of which are of only theoretical significance at present, will occur only after prolonged periods of time[18,19] and may be missed in preclinical studies with limited follow-up after genetic manipulation of HSCs. However, increasing the potency of the methods and the numbers of treatments may confront us with a growing number of reports.

Indeed, this review was prompted by our observation of a leukemia in a mouse study with prolonged follow-up after retroviral gene transfer into hematopoietic cells.[20] Unfortunately, the first case of a malignant disorder following clinical retroviral vector-mediated gene transfer into human hematopoietic cells was observed shortly thereafter, manifesting 3 years after the infusion of retrovirally modified cells[21,22] so that a once theoretical risk has become a real one. The uncertainty observed in the scientific and regulatory community following these reports[23,24] reflects a considerable need for systematic toxicology of genetic cell modifications.

Paracelsus, a founder of toxicology, has provided 3 golden rules for the assessment of side effects. The first is that poison is a question of dose.[25] Dose issues are encountered at several levels in hematopoietic gene therapy (Figure 2): the number of gene transfer particles to which the cells are exposed, the transgene copy number per cell, transcription rates, efficiency of RNA processing, protein features such as activity or stability of enzymes, the size of the target cell pool (generating a clonal repertoire due to the variations in transgene processing and integration), the life span of transplanted cells, and the number of patients treated.

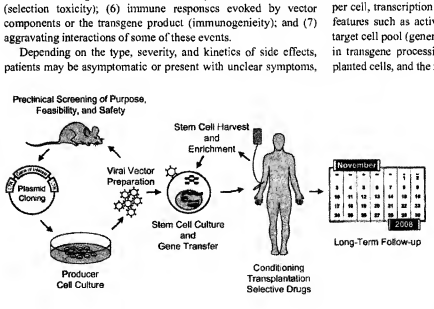


**Figure 1. Schematic overview of the procedures involved in the ex vivo manipulation of hematopoietic stem cells for gene therapy and preclinical approaches.**

Paracelsus' second rule is that a compound has a specific site (within the body) where it exerts the greatest effect.[25] Applied to gene therapy, this indicates that cell type and its developmental plasticity really matter. The third rule is to use animal models for preclinical dose finding.[25] Therefore, the limitations of animal models also have to be considered. Cell specificity and animal testing have been central items in gene therapy from the beginning. However, most studies focused on efficiency and were not designed to measure unexpected effects.

The present review summarizes recent insights into molecular mechanisms underlying side effects of genetic interventions in HSCs, following the classification of issues listed above (Table 1), and discusses consequences for the most commonly used animal model, the laboratory mouse.

## Toxicity of cell manipulation

Under steady-state conditions (normal hematopoietic turnover and an intact bone marrow niche), the majority of HSCs cycles slowly, yet continuously.[26-28] For genetic modification, HSCs are either harvested from peripheral blood or bone marrow.[29] The yield and biologic features of cells from these sources differ depending on the use of mechanical harvest versus cytokines (typically granulocyte colony-stimulating factor [G-CSF]) and/or chemotherapy, which may have direct implications for the efficiency of retroviral transduction and engraftment.[29-31] Exposure to cytotoxic agents may compromise the engraftment potential of HSCs.[32] Umbilical cord blood is a promising resource of stem cells, but the limited numbers of HSCs contained in cord blood may restrict a wider use in adults.[33,34]

Target cells of genetic manipulation usually have to be enriched to facilitate physical interaction with vector particles (Figure 1). Enrichment of HSCs for clinical use is most frequently achieved by immunoaffinity selection for the CD34 antigen. Developed for "mainstream" clinical applications, these processes for cell harvesting and enrichment have an excellent safety profile, and the engraftment potential of CD34-enriched cells is very good.[35] However, according to our current understanding, long-term repopulating HSCs probably represent less than 1% of the $CD34^+$ cell pool. Thus, the target pool size currently used for gene transfer is probably about 100-fold greater than actually required.

In theory, manipulating 10 000 HSCs (or maybe even much smaller numbers) should be sufficient to achieve a polyclonal transgenic hematopoiesis.[27,36,37] This would reduce significantly the numbers of vector particles required for cell manipulation, the risk of random mutagenic events that are related to the number of transgene insertions (below), and probably also the costs of the procedure. However, methods required for further enrichment of HSCs, such as isolation of the $CD34^+CD38^-$ population or their more primitive precursors,[29,36-40] have not yet been established for routine clinical use. High-grade purification of HSCs based on flow cytometry sorting has been shown to be feasible, but concerns remain regarding the risk of contamination, fitness of the sorted cells, selective interference with short-term engraftment, and risks associated with cell expansion.[39]

Although short-term reconstitution may be promoted following cell expansion in vitro,[41] current culture conditions may induce a selective loss of long-term HSCs.[29] Several underlying mechanisms have been identified: commitment to differentiation (loss of pluripotency) or even apoptosis, a cell-cycle–associated loss of engraftment/homing properties, and differential susceptibility to natural killer cell–mediated rejection.[29,42-44] Although engraftment with cultured cells alone has been rapid and sustained in clinical gene therapy studies,[45,46] extended manipulations, such as prolonged culture or enrichment of cells expressing the transgene prior to infusion, may promote deficits in long-term reconstitution.[29,41,47] Similar considerations apply for lymphocyte cultures.[48] Long-term follow-up, which in humans encompasses many years, will be required to draw firm conclusions that HSC exhaustion is not triggered by the procedures used during HSC manipulation in vitro.[29] Therefore, all efforts invested to maintain stem cell properties during in vitro culture are important. Improvements of HSC culture can be achieved by (1) the use of serum-free culture conditions,[49] (2) the definition of appropriate cytokine combinations,[50] (3) the manipulation of transcription factor levels such as HOXB4,[51] (4) the introduction of other (such as extracellular matrix) molecules[52-54] or appropriate stroma components,[55,56] and (5) protocols allowing a return to cell-cycle quiescence prior to infusion.[57,58] Moreover, new vector systems are being developed to reduce the need for stem cell proliferation prior to gene transfer.[13,59-61]

It may also be interesting to expand engineered cells in vitro following gene transfer. However, in at least one case, this attempt has been associated with an increased risk of malignant transformation of transduced murine hematopoietic cells.[62] Although it is possible that the expansion culture promoted a specific side effect of the vector or packaging cells used in this study, further work is required to address the extent to which culture conditions support a preferential growth of mutants with proto-oncogenic lesions.

In summary, new procedures for HSC harvest, enrichment, gene transfer, and expansion culture need to be studied intensively before clinical application. Besides "conventional" mouse models,[32] immunodeficient mice[38,63] or fetal sheep[47] supporting engraftment of primitive human hematopoietic cells and supporting studies in nonhuman primates[64] serve as valuable models for this purpose.

## Risks related to vector production

Conventional retroviral vectors based on mouse leukemia virus (MLV) and the more recently developed lentiviral vectors (such as those based on HIV-1) differ in many respects, particularly in their nuclear import strategies.[13,60,61] While MLV vectors require cell division for chromosomal insertion, lentiviral vectors may also transduce nonproliferating cells. However, lentiviral transduction efficiency also declines according to cell-cycle stages in the order $M > G_1 > G_0$. Another feature of HIV-based lentiviral vectors is that complex transgene cassettes containing cryptic splice sites are more reliably transferred,[65-68] which may be related to regulatory functions of the viral REV protein expressed during the packaging process. Because of the significant differences in the biologic properties of the viral proteins involved in the generation of replication-defective vectors, MLV and HIV vectors have distinct requirements for the design and culture of their respective producer cells.

### Stable producer clones are more easily established with long terminal repeat (LTR)–driven vectors

Progress in the design of retroviral vectors developed on the basis of MLV has improved their performance with respect to vector production, gene transfer efficiency, and transgene expression.[19,69-71] This vector system is the only one currently used in

clinical trials for stable gene transfer into HSCs.[2] One important advantage of MLV packaging components is their lack of cytotoxic effects, resulting in the ability to derive defined cloned producer cell lines with only 1 or 2 transgenes ("provector") inserted to encode the vector. This ensures the highest degree of transgene stability that can be achieved with retroviral packaging cell technology. However, the establishment of stable cloned producers with a limited and defined transgene copy number is greatly alleviated when retroviral gene transfer is used to establish the provector. Thus, transcriptional control needs to originate from the long terminal repeat (LTR), as in wild-type retroviruses. This configuration implies an increased risk of activating neighboring cellular sequences in target cells (below) and for recombination with viral coding sequences in the packaging cells, potentially facilitating the accidental generation of replication-competent retrovirus (RCR) recombinants.[16]

### Replication-competent retrovirus

Contamination of vector stocks with RCR can be detected by polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), or cell biologic assays. While the sensitivity of these methods can be very high, residual contamination of a clinical vector preparation as a matter of principle cannot be fully excluded. Important improvements in the design of vectors and packaging cells have greatly reduced the risk of generating RCR.[69]

The risk of developing a disease following accidental exposure to an MLV-related RCR depends heavily on the genetic background of the recipient and the integrity of the immune system. Replication-competent MLV with an amphotropic envelope protein was not found to represent a significant pathogen for immunocompetent or transiently immunosuppressed nonhuman primates.[19] However, when CD34$^+$-enriched cells were exposed to high titers of RCR-contaminated vector preparations in vitro and infused under conditions of strong immunosuppression, rhesus monkeys developed lymphomas within one year.[72] This required the absence of an immune response against retroviral particles or infected cells, and was likely driven by insertional mutagenesis (below) due to massive virus replication within susceptible lymphoid cells.[19,72] When inoculated into newborn mice, amphotropic MLV may also induce a spongiform eneephalopathy, whose kinetics and anatomic distribution depend on the type of the envelope protein.[73]

Potential RCR originating from lentiviral packaging cells has not been described to date. Accordingly, the potential pathogenicity of such recombinants is unknown, yet expected to be unlike that of wild-type HIV as a result of the anticipated differences in Env proteins, regulatory elements for gene expression, and the absence of many HIV accessory genes. An established limitation of currently used stable lentivirus packaging lines is genetic instability, because they may undergo multiple superinfection events when cultured (due to the lack of subgroup interference with the VSV-G pseudotype used).[74-76]

Although no side effects have been reported in more than a decade of clinical experience even with early generations of retroviral producer cells,[77] stringent safety testing and further technological improvements are still desirable for both retro- and lentivirus production systems. In the unlikely event of accidental exposure to RCR and their escape from immune control, it may be possible to suppress viral replication in patients using clinically approved inhibitors, unless resistance develops.[78]

### Mobilization

In the absence of an RCR originating from the packaging cells, spread of a retroviral vector could be possible when naturally occurring viruses exist that can package the vector RNA and are transmitted in the human population. This concern appears to be irrelevant for MLV vectors,[79] but needs to be considered for vectors developed on the basis of HIV or other lentiviruses.[80] To prevent this problem, lentiviral vectors are typically designed with a so-called self-inactivating (SIN) LTR. This is achieved by placing the enhancer-promoter into an internal position between defective LTRs, eliminating transcription of the packaging signal required for incorporation of the vector RNA in virus particles.[60,61]

### Transient transfection for vector production

So far, both lentiviral and previously investigated retroviral SIN vectors cannot be produced at sufficient titers from cloned packaging cell lines.[74] Efficient production of SIN vectors has been achieved only following transient transfection of plasmid vector constructs into packaging cells.[61,74,81] Although significant amounts of vector particles can be produced using this procedure,[81] concerns remain unresolved regarding the type and incidence of plasmid recombinations, accidental transfer of plasmid DNA with vector particles,[70] and the identity of the product obtained in independent production batches.

### The infidelity of reverse transcription

A limitation common to all types of retroviral vectors is the possibility for transgene recombination or mutation occurring during the obligate step of reverse transcription. The retroviral enzyme reverse transcriptase converts RNA to double-stranded DNA with an infidelity of about $10^{-4}$, suggesting that mutations are introduced once per 10 kb of a retroviral RNA template.[82] This may reflect an evolutionary pressure to produce about one mutation per replication cycle, given a genome size of natural retroviruses in the range of 8 to 11 kb. The misincorporation rate is similar for vectors based on MLV and HIV.[83] If we consider as a worst case scenario a proto-oncogene such as *N-RAS* with a size of 570 bp, one mutation could occur per 18 retrovirally transduced copies. For *N-RAS*, at least 3 activating mutations are known from a total of 1710 ($3 \times 570$) possibilities for single-point mutations (http://www.expasy.ch/cgi-bin/niceprot.pl?p01111). Thus, about one oncogenic *N-RAS* mutant would be formed per $10^4$ reverse transcriptions. In a clinical setting, about $10^8$ to $10^9$ infectious particles are required per CD34$^+$ cell preparation. Therefore, it may be important to define the oncogenic mutation frequency for a given cDNA, especially when dealing with transgenes encoding "signaling" molecules.

Much more frequent errors in transgene replication may result from sequence deletions or recombinations before or during reverse transcription.[82,83] Regulatory genome sequences that can be required to achieve cell-type–specific gene expression[84] and some clinically relevant cDNAs such as *MDR1* or *HSV-TK* may contain cryptic splice sites that give rise to pregenomic splicing of the vector RNA in packaging cells.[85,86] Interestingly, the frequency of these cryptic splicing events also depends on the packaging cell line.[87]

Also, intrastrand or interstrand recombinations are not uncommon during reverse transcription (retroviruses typically package 2 copies of a pregenomic RNA). These can be triggered by direct sequence repeats within the transgene,[83,88] and again occur with

similar frequency in vectors based on MLV or HIV.[83] The vast majority of such events will simply reduce the efficiency of the gene transfer. However, it may be worthwhile to address potential hazards induced by aberrations of a given transgene prior to clinical testing. Attempts to reduce sequence repetitions, to eliminate unwanted splice sites, and to choose appropriate packaging cell clones greatly improve the fidelity of transferring intact transgene sequences.[84,87,89,90]

Another concern related to vector production is the accidental incorporation of cellular RNA in the retroviral particle. Acutely transforming retroviruses encoding cellular oncogenes have evolved through such events, again requiring recombination during reverse transcription.[82,91] However, to create such an unwanted oncogene vector, further mutations triggered by multiple rounds of replication in virus/vector spread are typically required. Therefore, this risk appears extremely low with a replication-defective vector.

## Risks related to transgene insertion

Complications resulting from transgene insertion (insertional mutagenesis) are a concern for all stable gene transfer methods. Retroviral insertion has some unique properties. The first resides in the fact that insertion is a default event in the retroviral life cycle,[82,91] implying that the frequency of transgene insertion per cell can be predetermined by adjusting the multiplicity of infection.[92] The second is that insertion tends to take place in euchromatin, possibly because of its improved accessibility.[93] Consequently, the risk for insertion in transcriptionally active regions of chromosomal DNA is increased, as recently also demonstrated for HIV and derived vectors.[94] This implies a possibility for a cell-type–specific distribution, also assisted by host factors that participate in the preintegration complex.[94] Retroviral integrases are not sequence-specific with respect to transgene insertion, yet prefer specific structural features (bended DNA).[95] Thus, some yet unknown genetic loci may be at increased risk for retroviral insertion.[94] The third important feature of retroviral insertion is that it typically does not create subsequent recombinations within or outside the affected locus, although exceptions to this rule have been reported. Post-integration deletions may occur within repeats present in a single retroviral transgene, but these events appear to be rare.[96] Mutations within and surrounding a retroviral genome during expansion or malignant transformation of a transduced cell have also been described.[97,98] Finally, recombinations may occur between sequence-related, yet independently inserted, retroviral alleles.[99] However, the incidence of such events in nontransformed cells is assumed to be low (although probably not as low as the error rate of the cellular replication machinery, which is in the range of $10^{-9}$ per base and replication). Compared with retroviral gene transfer, physicochemically transfected DNA, especially when forming transgene concatamers,[15,100] as well as AAV gene transfer[101] may be associated with increased risk of genetic instability, also involving flanking cellular sequences.

### Incidence of recessive and dominant oncogenic insertions

With improving sequence information available from the murine and human genome projects, retroviral insertion events become increasingly mappable with regard to their exact to-the-base chromosomal location, relation to neighboring sequences, and potential interference with coding and regulatory regions.[20-22,94]

Previous assessments of the risk of untoward side effects from retrovirus insertion have been estimated to be rather low (between $10^{-6}$ and $10^{-8}$ per insertion event).[97,102,103] In an experiment involving retrovirus infection of mouse embryonic carcinoma cells with a high copy number, the risk for inactivating a single gene locus (usually a recessive mutation) comprising 0.001% of a murine genome was determined to be in the range of 1 to $4 \times 10^{-8}$ per insertion.[97] The risk for producing a phenotype that could also be induced by dominant activation of oncogenes (growth-factor independence in TF-1 human leukemia cells) was in the range of $2 \times 10^{-7}$ per insertion.[102] However, these experiments focused on specific transforming events or mutation of specific target genes and involved cloning procedures to identify mutants, possibly reducing the sensitivity of the detection systems.

Based on the hypothesis of semirandom choice of target sequence, proto-oncogenic activation by a transgene insertion event would be expected to be more frequent. Considering that the entire human genome consists of approximately $3 \times 10^9$ base pairs (bp), a transforming insertion event frequency of $10^{-7}$ would mean that only a few hundred base pairs in the entire genome would allow oncogene activation. In the light of the fact that wild-type retroviruses have been demonstrated to interfere with genetic regulation from distances as far as 90 kbp upstream,[104] such numbers appear unrealistic.

Restricting the area of retrovirus insertion interference to a diameter of about 10 kbp around a given gene, the chance of a single insertion interfering with a defined allele is roughly $10^{-5}$. Between 100 and 200 proto-oncogenes or oncogenes have been "fished" from the murine genome by retrovirus insertional mutagenesis studies.[105,106] With a margin of safety, the number of potential proto-oncogenes in the human genome is therefore probably not higher than 1000. The risk of an insertional event within 10 kbp of a potential proto-oncogene can therefore be estimated to range between $10^{-2}$ and $10^{-3}$.

At least 3 layers of safety, however, prevent such insertion events from being directly cancerogenic: first, retrovirus vector insertion is almost uniformly monoallelic,[107] reducing the relevance of most recessive mutational events. This restricts the influence of insertional disturbance to the much more rare setting of dominant effects that are biologically active even if just one locus has been changed. Second, some signal alterations may trigger differentiation or apoptosis, impede engraftment, or otherwise reduce the survival probability of the affected cell clone. Third, and foremost, a single insertional mutation is, to our current knowledge, not sufficient to develop a malignant phenotype by itself.[108] In the vector-associated incidents of murine and human leukemia that have recently been described,[21,22] the insertional oncogene activation has at best contributed to a premalignant expansion of cells later developing the malignant clone because of additional genetic events. This underlines the need to screen for potential cooperation of insertional mutagenesis with side effects of the transgene or other circumstances contributing to clonal expansion of gene-modified cells (below).

An issue of unknown significance is whether multiple insertions in single cells will lead to a disproportionate increase in the risk for insertional mutagenesis, although the few available data suggest a linear relationship between insertion frequency and mutagenesis.[97,102] It cannot be excluded that a high copy number of largely identical retrovirus transgenes distributed all over the genome may trigger chromosomal instability. In general, side effects observed under conditions of a high multiplicity of infection[62,109] may not be relevant for a more carefully controlled transduction procedure.[110] Considering these uncertainties, it appears reasonable to opt for the transfer of not more than 1 or 2 transgenes per cell. This represents

the efficiency of currently available methods,[36,111] but may in the future be more of an issue in vector systems with a higher efficiency of integration or high multiplicity of infection.[112]

In conclusion, the likelihood of oncogenic lesions induced by insertional mutagenesis alone would be expected to be relatively small when compared with some other established medical treatments, such as irradiation or chemotherapy with DNA-damaging agents.[103] Transformation of non-stem cells initiated by insertional mutagenesis does not seem to occur frequently: before 2002, no severe side effects related to insertional mutagenesis had been reported in more than a decade of clinical experience with retrovirus gene transfer into more committed hematopoietic cells and mature lymphocytes,[1,2] probably involving the manipulation of more than $10^{12}$ cells. The number of cases in which these observations were made long-term is substantial, although the number of repopulating stem cells engrafted altogether probably did not exceed 10 to 100 per patient, putting the overall number of transgene insertion events in HSCs under long-term observation at approximately $10^4$ to $10^5$ worldwide.

### Impact of vector design

The LTR configuration of conventional retroviral vectors comes with an increased risk to activate neighboring cellular sequences. The LTR establishes the enhancer-promoter regulating initiation of transcription and the polyadenylation signal giving rise to its termination on both ends of the transgene (Figure 3). Although MLV enhancer-promoters are strongly active in most hematopoietic cells (albeit with pronounced differentiation dependence),[71,113] the polyadenylation signal is relatively weak.[114] Moreover, the major retroviral splice donor or related motifs in the transgene may interact with downstream splice acceptors of cellular genes. Combined with insufficient termination, these features generate a number of possibilities for activation of cellular sequences located downstream of the transgene insertion site (Figure 3).

Some of the mechanisms giving rise to activation of a cellular gene also apply to lentiviral or MLV vectors with a SIN architecture.[115] However, the most frequent mechanism involved in retroviral insertional oncogene activation appears to be enhancer related, possibly working orientation independent and over large distances. Such a risk applies to almost any type of transgene configuration.

Considering the molecular mechanisms underlying activation of cellular genes, one could design vectors of improved safety. Such a vector should have a strong RNA termination/polyadenylation signal (serving as an "RNA insulator")[114,115]; an internal position of the enhancer and promoter sequences that are excluded from functional interactions with neighboring sequences through the inclusion of dominant DNA insulators[116]; and a strong internal splice acceptor that largely prevents interaction of the retroviral splice donor with downstream sequences. If functioning as predicted, such a (hypothetical) construct depicted in Figure 3B would reduce the risk of insertional mutagenesis to the residual risk of disrupting genes. The latter may often be irrelevant unless haploinsufficiency becomes phenotypically relevant or loss of heterozygosity occurs through independent hits.

Another strategy to avoid insertional mutagenesis would be to achieve targeted insertion of transgenes into predefined "benign" cellular loci. Although conceptual progress has been achieved in the manipulation of retroviral integrase, experimental evidence for a stringent, sequence-specific targeting strategy is limited.[117] A recent report indicates that physicochemical transfection procedures may be developed for targeted transgene insertion into defined genome loci in vivo (murine hepatocytes).[16] It remains to be seen whether such technologies are free from genotoxic side effects and how they can be adapted to HSCs. Similar considerations apply to targeted transgene insertion technologies developed on the basis of AAV.[118]

Besides these primary prevention strategies, vectors could also be equipped with selectable marker genes to generate options for secondary prevention strategies. A drug-resistance marker could be used to reduce the clonal repertoire in vivo (Figure 3C) by ablating cells with low expression levels.[119] However, if insertional oncogene activation enhances the fitness of cells during selection, this strategy may be counterproductive. Experiments addressing this issue have not been reported to our knowledge. Another option would be to include a negative selection marker in the transgene cassette. A conditional suicide gene (such as *HSV-TK*)[80] may help to eliminate a malignant clone (Figure 3D), especially when combined with other antineoplastic treatments. However, this would also result in the loss of nontransformed transgenic cells. Before such an approach can be recommended, the potential

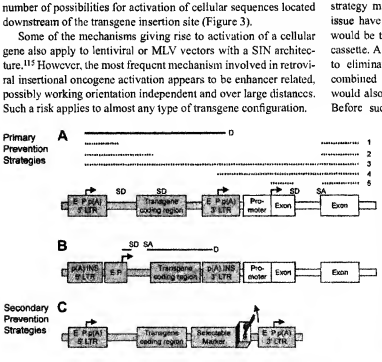


**Figure 3. Impact of vector design on the potential activation of a cellular gene located downstream of the transcriptional direction of the vector.** The gray boxes in panel A show a randomly inserted retroviral transgene with a conventional LTR architecture. Here, the enhancer (E) and promoter (P) are terminally repeated, and the polyadenylation [p(A)] signal is weak; a splice donor is present in the retroviral untranslated region and another one within the transgene cDNA. The desired vector transcript is shown as line D; potential aberrant transcripts are numbered and shown as dotted lines. Either of the vector's SDs may interact with a splice acceptor (SA) of a downstream-located cellular gene to generate alternative splice products 1 and 2. Aberrant transcripts 1 to 3 result from lack of termination; transcript 4, from activation of the 3′ promoter of the vector; and transcript 5, from a distant action of the vector-enhanced on a cellular promoter (which may also be located upstream and/or in inverse orientation to the insertion). Transcripts similar to 1 and 4 have been detected in the case of leukemia following retroviral gene marking in mice.[20] The hypothetical vector shown in panel B was designed to prevent aberrant transcripts by deleting the enhancer-promoter from the LTR, inserting a strong splice acceptor upstream of the vectors coding sequences, deleting SD of the cDNA, utilizing a strong p(A) signal, and flanking the transgene cassette with insulator (INS) sequences that prevent long-distance enhancer interactions. Secondary prevention strategies shown in panels C and D make use of coexpressed selectable marker genes ("impact of vector design").

immunogenicity of many suicide gene products and the limited preclinical experience with introducing suicide genes into HSCs has to be overcome.

## Risks related to transgene expression

The ultimate goal of genetic therapy is to replace in situ a defective gene sequence, ideally by homologous recombination repair of the original locus. However, using available vectors and HSCs as targets, somatic gene transfer typically results in ectopic and nonregulated expression of the transgene, both with respect to the cell type affected and the level of expression achieved.

Depending on the type and assembly of cis elements used, expression levels generated by different vectors may differ by up to 3 orders of magnitude. Different variants of MLV enhancer-promoters and some cellular promoters have shown a great potential for multilineage and persistent transgene expression in hematopoietic cells in vivo, typically accounting for less than 1% of the total cellular protein content.[71,113,119-123] Cellular control elements have been modified to provide lineage-specific expression with promising potency,[65-67,124] and inducible expression has been achieved with designer promoters.[68]

The insertion site modulates all aspects of transgene expression, including duration, level, and differentiation dependence. With LTR-driven retroviral vectors, the majority of unselected clones shows fairly similar transgene expression levels. However, interclonal variability of transgene expression may be as high as 50-fold, and complete silencing can be observed in some HSCs and their progeny.[113,120,122] Unless targeted insertion into the correct cellular allele or specific regulation is achieved, transgene expression will hardly ever be physiologie in every transduced cell.

According to Paracelsus' first rule (poison is a question of dose), it can be predicted that any transgene product has a defined therapeutic window compatible with the desired function and without the predominance of unwanted effects. Toxicity related to transgene expression may most frequently manifest in a competitive disadvantage, leading to the extinction of the affected cell (clone) and thus to a loss of efficiency. However, transgene interference with cellular decisions related to homing, proliferation, or differentiation may eventually result in the manifestation of new types of diseases. Currently, few observations are available that support these concerns. However, we have to be aware that up to now far less than 1% of the human cDNA pool and a necessarily minute fraction of all artificial sequences possible have been introduced into gene therapy research. Moreover, gene delivery systems have and will continue to become increasingly potent, also allowing the simultaneous transfer of 2 or more cDNAs with a single vector.

To support these considerations, 4 examples may be sufficient. Of the 4, 3 deal with the use of selectable marker genes, a key technology in hematopoietic gene therapy. These examples provide evidence for dose-dependent toxicity (*HOXB4*), an as yet uncertain contribution to a severe side effect (*MDR1*), and evidence for context-dependent side effects (*dLNGFR*). These and a final example (*CD40L*) highlight the importance of developing vectors for spatially or temporally controlled expression of transgenes.

### Ectopic expression of *HOXB4*: dose-dependent side effects?

Retroviral vector–mediated expression of *HOXB4*, encoding a homeodomain transcription factor involved in the regulation of hematopoietic pool size, has been shown to promote polyclonal and regulated expansion of engineered HSCs.[51,125] In contrast to many other homeobox genes, ectopic expression of *HOXB4* in hematopoietic cells did not lead to overt alterations of differentiation or uncontrolled expansion of gene-modified cells in mice.[126] The interest in *HOXB4* gene transfer for cell therapy has been reinforced by the finding that murine embryonic stem (ES) cell–derived hematopoiesis can be partially converted to repopulation competence in adult hosts upon transient or stable activation of *HOXB4* expression.[127]

In human HSCs transplanted into immunodeficient mice, ectopic expression of *HOXB4* promoted the expansion of primitive hematopoietic cells.[128,129] However, high levels of HOXB4 expressed from "stronger" vectors impeded myeloid and lymphoid differentiation of human hematopoietic cells.[129] In line with these data, impaired repopulation of lymphatic tissues was observed in a study using *HOXB4*-engineered hematopoietic cells derived from a somatic cloning procedure.[130] These studies taken together argue that the effects of *HOXB4* are highly dependent on the dose and the kinetics of its ectopic expression. Importantly, activation of HOXB4-interacting partners such as *PBX1* (possibly by insertional mutagenesis) may be sufficient to promote transformation of HSCs with constitutive ectopic expression of *HOXB4*.[131] Thus, a potential therapeutic use of HOXB4 may require an exact definition of a therapeutic window and may depend on the ability of regulated expression.

### Murine leukemia following *MDR1* gene transfer: phenotoxicity, genotoxicity, or both?

Adenosine triphosphate binding cassette (ABC) transporter pumps encoded by multidrug resistance 1 (*MDR1*) or *ABCG2* are naturally expressed in primitive hematopoietic cells, explaining their inherent competence for extruding some fluorescent dyes and other amphiphilie compounds.[132,133] Increasing expression levels of such pumps may promote a survival advantage in the presence of high doses of some chemotherapeutic agents,[134,135] and independently antagonize some proapoptic signals, as shown for *MDR1*.[132,136,137]

Interestingly, ectopic expression of *ABCG2* was associated with impaired differentiation of myeloid cells in mice.[138] It is yet unclear to what extent this effect is dose related. The results with *MDR1* have been controversial. Numerous studies, including a transgenic mouse model, have shown the ability to overexpress *MDR1* in hematopoietic cells without overt alterations of cell functions (other than the acquired drug-resistance phenotype).[85,134,135,139,140] Applications in dogs,[141] nonhuman primates,[110] and clinical trials[45,46,142] have been safe, with occasional evidence for increased pump activity, although gene transfer efficiency was likely very low.

However, myeloproliferative disorders have also been observed in different strains of mice using retroviral vector–mediated transfer of *MDR1* into hematopoietic cells,[62,109] and disease induction was promoted by prolonged expansion of cells in vitro prior to transplantation.[62] Interestingly, this disease was associated not only with ectopic expression of *MDR1*, but also with an unusually high transgene copy number (in many cases exceeding 10 copies per clone, which is quite unusual even in mouse studies). Therefore, the most straightforward explanation is that excess *MDR1* expression in this study may have been pathogenic. Besides, sequences other than *MDR1* could have been expressed from insertion of intact or rearranged vectors. This aspect needs to be clarified, as the genetic integrity of the inserted transgenes has not been investigated, and the disease was so far observed only with a

**Table 2. Classes of selectable marker genes and corresponding drugs**

| Principle | Utility | Example for gene | Drug/agent category | Agent | Reference no. |
|---|---|---|---|---|---|
| Surface tag | In vitro | *dLNGFR* | Monoclonal antibody | NA | 120,121,123, 144-149 |
| Drug resistance | In vitro and in vivo | *MGMT* | Cytotoxic agent | Temozolomide, BCNU | 137, 150-152 |
| | | *DHFR* | Cytotoxic agent | MTX | 137 |
| | | *MDR1* | Cytotoxic agent | Paclitaxel, etoposide | 45,46,134,135,139-142 |
| Growth promoting | In vitro and in vivo | *FK-mpl* | Chemical dimerizer | AP1903 | 153,154 |
| | | *SAG* | Inducer of protein function | Estrogens | 155 |
| | | *HOXB4* | Controlled expression required | NA | 51,125-131 |

This table does not provide a comprehensive overview of the genes developed as selectable markers. Its purpose is rather to show the different principles with a few selected examples. NA indicates currently not available as good manufacturing practice product.

specific vector backbone (based on a first generation vector derived from Harvey murine sarcoma virus containing, in addition to an engineered "splice corrected" cDNA, considerable amounts of viral gene remnants that are not required for proper vector function).[62,109] Moreover, it has not been reported whether the otherwise well-designed control vectors used had a similar high copy number in the producer and target cells.[62,109] Thus, it remains formally unclear whether the disease was dependent on side effects of very high *MDR1* expression (driven from multiple transgenic alleles in the mouse model);[137] the expression of vector sequences other than *MDR1* (potentially driven from rearranged vectors); or an increased risk for insertional mutagenesis or genomic instability under conditions of high copy numbers per genome. It is also quite possible that some or all of these factors acted together to produce the myeloproliferation.

Another open question is whether *MDR1* overexpression may promote engraftment of gene-modified HSCs,[45] although *MDR1* expression alone would not be sufficient to overcome a culture-dependent loss of engraftment capacity.[143] Taken collectively, these data indicate that defining a therapeutic window for ectopic expression of *MDR1* or other efflux pumps in hematopoietic cells may be difficult. If future research will not facilitate the definition of safe conditions of transgenic *MDR1* expression, alternative metabolic selection markers may be more promising (Table 2 and references therein).

### Context-dependent toxicity of a cell-surface marker: *dLNGFR*

The cytoplasmically deleted low-affinity nerve growth factor receptor (*dLNGFR*, also abbreviated Δ*LNGFR*, *LNGFR*, *tNGFR*, or *NGFR*) was derived from p75 neurotrophin receptor (*p75NTR*) to develop a clinically applicable cell-surface marker for hematopoietic cells.[144] Although *dLNGFR* has been used by several laboratories to tag gene-modified cells,[145] few data have been published regarding the ability for long-term marking (> 1 year) of HSCs and their progeny.[146] A nonhuman primate study reported a failure to mark long-term repopulating HSCs with *dLNGFR*, however without investigating potential mechanisms.[147] On the other hand, use of *dLNGFR* in clinical trials with gene-modified T cells has been shown feasible and safe.[148,149] However, a recent mouse experiment[20] in conjunction with an earlier study in fibroblasts[150] offered the hypothesis that *dLNGFR* expression in myeloid cells may promote their transformation in an unusual, highly context-dependent manner. It is this proposed context dependence that renders the discussion of this issue interesting.

p75NTR is a member of the tumor necrosis factor (TNF) receptor superfamily that can bind all known neurotrophins (NTs) including nerve growth factor (NGF).[157] *p75NTR* is usually not expressed in hematopoietic cells, with the exception of some B-cell subsets.[158] The cytoplasmic domain of p75NTR contains a proapoptotic juxtamembrane region and a death domain.[157] These sequences were deleted in dLNGFR before their precise function was known in an attempt to create an inert surface marker.[144] The deletion may weaken the anchoring in the cell membrane, and therefore the shedding of dLNGFR,[145] which is still able to bind NTs in vivo,[151] may affect the local extracellular cytokine milieu. Moreover, deletion of the intracellular domain renders dLNGFR structurally similar to naturally occurring antiapoptotic decoy receptors of the TNF-receptor family, which can act as dominant-negative inhibitors of proapoptotic intracellular pathways.[160]

In cells expressing *TrkA*, *TrkB*, or *TrkC*, which encode tyrosine kinase receptors for different NTs, association of p75NTR creates a heterodimeric receptor complex with increased ligand affinity that is not dependent on the presence of the cytoplasmic residues.[157] It is noteworthy that coexpression of either one of the *Trk* receptors with a *p75NTR* mutant that lacked most of the intracellular domain, a construct basically identical with *dLNGFR*, resulted in transformation of fibroblasts when NTs were added to the culture.[156] This growth-promoting role dLNGFR is clearly dependent on the coexpression of a *Trk* gene and the presence of NTs (Figure 4). The same configuration occurred in the murine monocytic leukemia originating in association with retroviral insertional up-regulation of *Evi1* in hematopoietic cells, which provides circumstantial evidence but no formal proof of a contributing role of *dLNGFR*.[20]

*Evi1* encodes a Zinc-finger transcription factor that has been implicated in the pathogenesis of human myelodysplastic syndromes and acute myeloid leukemia (AML). Ectopic expression of *Evi1* impairs granulocytic differentiation, but leads to only mild alterations of hematopoiesis in transgenic mouse models.[161] We proposed a specific interaction of dLNGFR and Evi1 in the induction of the leukemic clone, possibly reflecting a bias for a

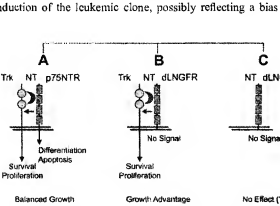


**Figure 4. Proposed context-dependence of side effects elicited by dLNGFR.** Situation A represents a physiologic situation that can be observed in neuronal and some other cell types.[157] Situation B was shown to promote the transformation of fibroblasts in vitro.[156] Situation C represents the ideal context for cell marking.[144]

lineage (ie, monocytic) in which *TrkA* expression and NGF signaling were also present and functionally relevant.[20,158]

If this hypothesis can be confirmed, it would represent an example for cooperation of random insertional mutagenesis (genotoxicity) and transgene-related side effects (phenotoxicity) in the induction of leukemia. Alternatively or in addition, Evi1 may have induced expression of *TrkA*,[162] and the interaction with dLNGFR may have promoted the transformation of a monocytic precursor. Also, a protein related to Evi1 has been shown to play a role in Trk-signaling of C elegans,[163] opening further possibilities for transforming loops.

The potential risk associated with the use of *dLNGFR* in HSCs is underlined by observations that signals generated through oncogenic versions of Trk receptors may contribute to the pathogenesis of human AML.[164-166] Therefore, *dLNGFR* does not appear to be a perfect choice for the manipulation of cells with a broad plasticity such as HSCs. However, as side effects of dLNGFR are proposed to be context dependent, its use in restricted cell lineages lacking cooperating signal transducers can be justified (considering Paracelsus' second rule). Interestingly, variants of dLNGFR have been developed that are deficient in ligand binding[167] in order to reduce the probability of side effects. Similar concerns of context-dependent side effects and potential for cooperation with randomly activated oncogenes apply to many other therapeutic or marker genes.

#### Problems resulting from unregulated expression: *CD40L*

Finally, the mode of transgene expression is an important determinant of potential toxicity. This has been exemplified in an attempt to develop gene therapy for inherited deficiency of the CD40 ligand (X-linked hyper-IgM syndrome). Ectopic constitutive, but not naturally regulated, expression of CD40L, although at low level, produced abnormal proliferative responses in developing murine T lymphocytes, apparently through dysregulated intercellular interactions during thymic maturation and selection.[168] For many applications of hematopoietic gene therapy it is worth repeating the conclusion of this study: "Current methods of gene therapy may prove inappropriate for disorders involving highly regulated genes in essential positions in proliferative cascades."[168]

These 4 examples should be sufficient to underline the importance of a systematic risk assessment of the transgenes under consideration. Special attention should be paid to molecules that are involved in cellular signaling networks, such as those required for correction of some inherited disorders[3,168,169] or those generated as surface tags[120,123,144] or artificially inducible proteins that promote cellular proliferation or differentiation decisions.[153-155] We would propose that such transgenes should be tested under conditions of high, intermediate, and low constitutive expression,[129] preferentially achieved with vector design and not with variation of transgene dosage. Preclinical assay systems available for such work range from cell-culture–based model systems to animal studies and functional genomics or proteomics (Figure 5).

## Risks related to conditioning, use of selective drugs, and cell amplification

Following genetic modification of HSCs in vitro, their engraftment and contribution to hematopoiesis in vivo are dependent upon the methods used for conditioning or selective amplification. Conditioning eliminates host cells prior to infusion of gene-modified cells.

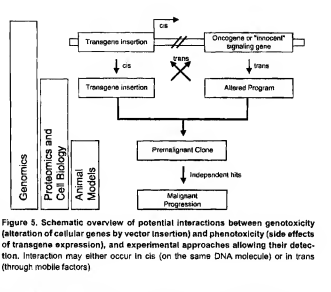


**Figure 5. Schematic overview of potential interactions between genotoxicity (alteration of cellular genes by vector insertion) and phenotoxicity (side effects of transgene expression), and experimental approaches allowing their detection.** Interaction may either occur in cis (on the same DNA molecule) or in trans (through mobile factors)

Irradiation or cytotoxic agents induce a moderate to severe (myeloablative) lymphohematotoxicity. However, these regimens can be complicated by severe long-term toxicity. Nonmyeloablative regimens with sublethal toxicity have become increasingly well investigated[170] and begin to show great promise for HSC-mediated gene therapy.[4,171] In animal models, high doses of donor cells[32] and application of G-CSF to the recipient before nonmyeloablative conditioning[172] have been shown to promote engraftment. However, it is unclear whether chimerism will be maintained in a stable manner in the long term when nonmyeloablative protocols are performed in an autologous clinical setting. Here, donor-dependent immune functions have no facilitating role to promote engraftment of the transplant; tolerance may be incomplete and engineered cells usually do not have a spontaneous selective advantage. An alternative, potentially more specific and thus less toxic approach to conditioning is the use of monoclonal antibodies directed against stem cell antigens or more common leukocyte antigens.[173] Although it is likely that side effects associated with conditioning regimens will be reduced significantly in the near future, this issue will continue to be an important aspect of the risk-benefit evaluation for stem cell–based gene therapy.

Importantly, several diseases could be successfully treated with a moderate rate of chimerism (5%-30%). A selective survival advantage of engineered HSCs can be promoted upon transfer and expression of appropriate selectable marker genes. Table 2 summarizes 3 different categories of such genes that have a well-documented efficiency in animal models. Potential side effects resulting from the expression of selectable marker genes have been reviewed above ("Risks related to transgene expression").

For most of these selectable marker genes, drugs are required to trigger their function. Therefore, side effects associated with these drugs represent another important aspect of the preclinical and clinical evaluation. Some of these agents have a well-documented toxicity profile in humans; others represent experimental agents with limited clinical experience. In this context, it is interesting to note that the most powerful selection system currently available for gene-modified hematopoietic cells requires the use of DNA-damaging agents.[137,150-152] Although potentially less toxic alternatives for selective amplification of gene-modified cells have been proposed,[153-155] expansion of hematopoietic cells promoted by these gene functions may be incomplete, lineage restricted, and unstable, suggesting preferential action at the level of progenitor cells as opposed to HSCs. This implies a need for repetitive use of

the corresponding drugs over prolonged periods of time, or induction of a distorted hematopoiesis with unclear long-term consequences.

Clonal amplification of transgenic cells is another important variable.[7] The risk for accumulating mutations that are not related to gene transfer increases with the life span and the number of generations of the engineered cell. In most conditions of human bone marrow transplantation, the size of the graft's stem cell dose implies a modest pressure for expansion and a high likelihood for polyclonal reconstitution. This is underlined by results from nonhuman primate studies of gene-marking.[36,111] With the advent of genetic selection strategies, a risk related to forced expansion of individual clones may become more relevant. On the other hand, single clones of transduced HSCs may provide a perfectly normal hematopoiesis with persistence of transgene expression in all hematopoietic lineages, at least in mice.[120,123] This supports the idea that clones with favorable insertion sites and "neutral" transgenes are not necessarily at increased risk for transformation, even when undergoing massive expansion. The minimal number of HSCs that stably support primate hematopoiesis remains to be defined.

## Risks related to immune surveillance

A further category of side effects is related to innate or acquired immunity against vector components or immune surveillance of engineered cells. A recent review proposed that certain gene transfer procedures may set "danger" signals that result in an increased likelihood of an immune reaction.[174] However, severe inflammatory reactions elicited by viral proteins in the vector preparation, as observed with early generations of adenoviral vectors administered in vivo,[175] are unlikely following a single administration of ex vivo–manipulated hematopoietic cells. In principle, a transient exposure to antigens may be caused by remnants of vector particles or culture media components on infused cells even if the transgene does not encode viral antigens.[176] This risk appears small with conventional retroviral transduction protocols in which cells are cultured for at least a day following the final exposure to vector particles. However, with the use of adenovirus[177] or lentivirus vectors[178] the time in culture after the final round of vector exposure may be shortened, which could increase the probability of contamination with viral antigens.

Repetitive infusion of engineered cells may be complicated by sensitization to antigens originating from components of culture media, vector particles, or transgene expression, potentially resulting in clearance of transgenic cells[179] or even severe acute adverse reactions. This potential problem could be solved by appropriate preparation of cells and recipient prior to infusion or simply by single use of engineered cells (given that the vector system does not generate antigens to which pre-existing immunity exists). Also, sensitization could be diagnosed prior to repeated infusion of cells.

Moreover, it will be interesting to determine whether some clinical settings, such as those resulting from repeated infections, may trigger cellular or humoral innate immune functions to clear incoming gene-modified cells or create an unfavorable cytokine milieu. If so, a transient blockade of these mechanisms, as proposed in the context of preclinical adenoviral gene therapy,[180] may improve the "take" of gene-modified cells.

Immune responses mounted against transgene antigens may develop with some latency. This concern is of particular relevance when introducing artificial or xenogenic sequences (as in the use of some selectable marker genes) and when correcting inherited genetic disorders in so-called CRIM (cross-reactive immunologic material)-negative patients. Although bone marrow transplantation may promote tolerance to multiple or individual antigens,[181-183] this does not necessarily occur following nonmyeloablative conditioning regimens. Immune-mediated rejection of transgenic cells expressing the xenogenic marker enhanced green fluorescent protein occurred in a study with nonhuman primates.[184] Disturbingly, one affected animal developed hemolytic anemia after rejecting the transgenic cells.[184] Further investigations are needed to determine whether autoimmunity can be induced as a side effect of sensitization against transgenic cells. For more advanced applications of gene therapy as well as for allogeneic transplantation, tolerance induction is a key issue of future research.

## Combination and interactions of risk factors

After listing this collection of potential problems, it is important to mention that combinatorial side effects of transgene insertion (genotoxicity), transgene expression (phenotoxicity), and cell expansion (selection toxicity) may be required to produce malignant transformation. Monocytic leukemia observed after *dLNGFR*-marking in mice and serial bone marrow transplantation may serve as a paradigm.[20] It is possible that at least some clones observed in the *CD40L*-induced lymphoproliferation[148] or in the *MDR1*-associated myeloproliferative disease[82,109] had a similar history involving insertional mutagenesis in addition to transgene side effects and/or forced cell expansion. Insertional activation (in cis) of an oncogene or an otherwise "innocent" transcription factor may change the cellular program (in trans), which in turn may cooperate with the transgene product to induce an undesired phenotype (Figure 5). Such program alterations may also influence expression levels of the transgene, jointly acting to promote the initial survival of a premalignant clone.

Similar considerations apply for the serious adverse event recently observed in a clinical gene therapy trial.[21,22] Ten children with X-linked severe combined immunodeficiency (X-SCID) were successfully treated by retroviral transfer of the interleukin 2 receptor common γ-chain into CD34$^+$ cells and reinfusion of cells without conditioning. This transgene was required to correct the underlying genetic deficiency and provides (in X-SCID patients with this deficiency) a powerful selective advantage during T-cell maturation.[3] The initial outcome of gene therapy was better than that typically achieved with allogeneic bone marrow transplantation.[3] At 3 years after cell infusion, one patient presented with clinical signs of an acute lymphoblastic leukemia (ALL) caused by a monoclonal proliferation of γδ T cells. The clone had one insertion of an intact vector copy that occurred in one *LMO2* allele (readily identified by LAM-PCR)[21,36] inducing ectopic expression of this proto-oncogene. *LMO2* is known to be involved in the pathogenesis of ALL, but probably not sufficient to cause malignancy (for which 4 to 6 independent genetic "hits" seem to be required).[21,22,108,185] Although no evidence for aberrant signaling through the transgenic common γ-chain has been reported, its physiologic function obviously was sufficient to promote a strong expansion of the clone. Although no detailed analysis is available at this point, it can be postulated that additional genetic hits could have subsequently promoted malignant progression: the malignant clone (expressing a mature T-cell phenotype) was more mature than the initially transduced cell population (which must have

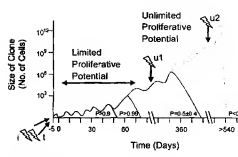


**Figure 6. Not every insertional oncogene activation is expected to result in clinical manifestation of malignancy (hypothetical curve).** Transformation-promoting events are symbolized by flashes; the one marked with I represents insertional mutagenesis; the one labeled t indicates transgene side effects; u1 and u2 indicate subsequent unrelated hits. Clone size, time course, and the probability of extinction represent arbitrary values.

lacked T cells), and a severe varizella zoster virus infection was reported to have coincided with first clinical symptoms of hematologic abnormalities. Moreover, a cytogenetic abnormality was observed that did not involve the insertion site. Another level of concern was a genetic background of childhood cancer.[21,22]

Given that an unfavorable concert of oncogenic factors is required for tumor manifestation (as in the model presented in Figure 6), such a serious adverse event is not expected to be found in any clinical scenario for retroviral gene therapy, nor can we predict its frequency in gene therapy for X-SCID patients. Besides the specific clinical setting and features of the transgene (cDNA and regulatory regions), target cell features represent an important variable. These determine the overall susceptibility to gene transfer, which loci are open for transgene insertion, and how many of these may contribute to malignant transformation.[103] Further points to consider include the number of cell generations following gene transfer,[103] the expansion conditions that may sometimes suppress balancing proapoptotic signals, the exposure to mutagenic hazards that are independent of the genetic manipulation, and the endogenous capacity for DNA repair and proliferation control. Finally, systemic responses to transformed cell clones add another level of complexity (Table 3).

Bearing this in mind, we need to consider appropriate preclinical models, including animal experiments, in order to derive clear statistics indicating the importance of individual risk factors and the probability of their combinations. For scientific, economical, and ethical reasons, studies will often rely on work with cell lines and laboratory mice. In this respect, it is important to discuss features of the mouse model that distinguish it from human hematopoiesis.

## The mouse model compared with human hematopoiesis

The outstanding role of the laboratory mouse for modeling human development and disease has received further support by the recent findings of the mouse genome project.[186] Nevertheless, the differences between the hematopoietic systems of mice and humans must be carefully evaluated to diagnose with certainty reactive and neoplastic blood cell disorders and to improve the predictive value of the animal model.

Even in humans, the classification of preleukemic states such as the myelodysplastic syndromes is still controversial.[187] Very recently, preleukemia and its progression to leukemia have been classified in mice,[188,189] in analogy to the French-American-British (FAB) scheme developed for human leukemia.[190] Murine leukemias may be experimentally induced with specific genetic alterations.[191] Examples for preleukenic alterations are ectopic expression of *BCR-ABL*,[192] *N-RAS*,[193] *BCL2*,[194] or *Evi1*,[161] and the *ICSBP*-knock-out mouse.[195] The latency period between leukemia induction by application of x-rays and/or inoculation of MLVs and leukemia manifestation could be regarded as a preleukemic condition.

The susceptibility of mice to develop leukemia varies according to strain and its contamination with MLVs. Unless a genetic predisposition is involved (such as endogenous RCRs), spontaneous leukemia occurs only sporadically in older animals. It can be induced with high incidence by irradiation and inoculation of newborn or immunodeficient animals with MLVs. RCRs transform cells by insertional mutagenesis, which has been useful in the identification of tumor-associated genes.[82,91,104-106,196,197] In some cases acute leukemia can be induced following a rapid polyclonal expansion of progenitor cells when a mouse is infected with a retrovirus complex that cotransfers a replication-defective retrovirus encoding an oncogene.[82,91]

The mouse has a significantly higher daily hematopoietic cell turnover (especially of red blood cells and platelets) compared with humans. Accordingly, the complete bone cavities are used for hematopoiesis, and there are very few or no fat cells interspersed.[198,199] Reactive or malignant increases of hematopoietic tissue rapidly lead to extramedullary hematopoiesis, typically starting in the spleen.[200-202] Thus, splenomegaly with expansion of red pulp caused by leukemic infiltrations (> 20% blasts) and regression of the white pulp (periarteriolar lymphatic sheath and lymph follicles) are characteristic and early findings in murine leukemia.[188,190] In advanced disease, normal architecture of spleen is totally abolished and widely replaced by masses of blasts. Liver

**Table 3. How cell type may determine the risk of insertional mutagenesis**

| Level | Before insertion | After insertion |
|---|---|---|
| Genome | Number of pre-existing mutations | Potential for secondary mutations |
| | Number and size of activated, potentially disruptible "dangerous" loci susceptible to transgene insertion | Proliferation capacity/propensity |
| Cell-transgene interactions, direct | Host factors participating in preintegration complex | Recognition of transgene regulatory elements and expression level of transgene |
| | Cell-cycle status | Type and number of mechanisms supporting transgene side effect |
| Cellular response, indirect | | Response balance to "danger" signals |
| | | Potential for escape from senescence |
| | | Number of generations following genetic modification |
| | | Level of differentiation |

involvement in AML is characterized by leukemic infiltrates in periportal areas. Therefore, unlike human leukemia, bone marrow is only variably, and spleen is constantly, involved in murine leukemia.[200]

The mouse model may not be fully predictive for human leukemia development when considering differences in HSC turnover. Leukemia development often involves genetic alterations of true HSCs.[5] However, it is unclear whether this also applies to oncogenesis related to retroviral manipulations. The pool size of murine HSCs is tightly regulated, although with considerable genetic and age-dependent variability.[203] Abkowitz et al postulate a similar size of the HSC pool in mice and cats (approximately 12 000 per animal), and possibly also in humans.[27] The study also suggests a conservation of the replicative activity per lifetime between murine and human HSCs.[27] Thus, mice would present with a higher density and shortened cycling times of HSCs within the bone marrow.

However, if the burden of insertional mutagenesis also involves less primitive progenitor and precursor cells, typical mouse experiments performed with a relatively small total number of hematopoietic cells would underestimate the risk (about $10^6$ transplanted per mouse, compared with at least $10^8$ cells in a clinical trial). Moreover, the life span of this animal is short (2 years), and for practical reasons, observation periods rarely exceed 6 to 12 months, further reducing the chance to detect slowly developing dysplasias. Bone marrow transplantation and inappropriate cell culture strongly reduce the pool size of HSCs.[81] Serial bone marrow transplantations generate monoclonal or oligoclonal hematopoiesis in mice, suggesting an enormous pressure for massive amplification of individual HSCs.[81,120,123,204,205] Such a forced expansion may promote the manifestation of dysplastic or overt leukemic clones.[7,206,207] Thus, although the mouse model suffers from a poor sensitivity to detect rare mutagenic events related to integrating, nonreplicating vectors, in case such events do occur, experimental conditions can be adjusted to promote their manifestation.

It has been demonstrated that rodent cells can be more easily transformed than human cells, and several functional differences of signaling pathways involved in human and rodent models of transformation have been identified.[108] One has been linked to a stronger activity of the telomerase function in mature rodent cells. Thus, only 2 to 3 hits are typically required for transformation of a rodent cell: 1 that dysregulates apoptosis and 1 or 2 further hits that alter cell-cycle control and provide proliferative stimuli. In humans, yet another event promoting telomerase activity is required for malignant transformation of mature epithelial and fibroblastic cells, but it is still unclear whether this would be needed to transform a human HSC.[5,108] If so, human cells would be more resistant to transformation by random vector insertions and transgene side effects.

Summarizing these considerations, it appears justifiable to develop mouse models in which the sensitivity for detecting side effects related to genetic manipulations is increased by the choice of the experimental conditions, such as in vitro expansion[62]; hemolysis[202] or bleeding[206]; serial bone marrow transplantation[20,207]; or introduction of a proto-oncogenic genetic lesion.[161,191-195] Also, "humanization" of the mouse genome may be helpful to obtain models of increased predictive value.[108,186] Currently available "large"-animal models would be even more relevant for human gene therapy, but obviously do not allow broader genotoxicity screening.[64] Moreover, tumor manifestations usually take many years in nonhuman primates. It may be better to design oversensitive mouse models as a "worst case" scenario, thereby generating clear statistics that reflect the impact of defined risk factors.

## Conclusions

Successfully exploiting the enormous potential of gene therapy targeting hematopoietic cells requires an open eye for side effects. Proof of principle in animal models may be spectacular, but is not all that is relevant for developing safe clinical applications. As with the use of drugs or irradiation, dose finding is a required next step. One realistic option to improve safety without loss of efficiency is to translate better procedures for stem cell purification into clinical use. The resulting reduction in the target cell pool likely represents a straightforward way to reduce the risk of insertional mutagenesis. The associated long-term goal is to reconstitute hematopoiesis with just a few clones of genetically characterized transgenic stem cells. Beyond the issue of target cells, dose finding takes into account the interaction of multiple features in vectors, transgenes, and clinical scenarios, which can be reflected in the design of preclinical models. Improved vector design may result in greater target site specificity of insertion and a transgene composition that has a reduced risk of activating cellular genes. Another important goal is to derive a risk classification of transgenes and clinical scenarios, considering all conditions potentially contributing to side effects. This may help to avoid premature generalization of side effects that occurred under specific circumstances. A database of vector insertion sites and statistical analyses on the clonality of reconstitution will be key to understanding the dynamics of these processes. Results obtained in long-term follow-up, especially, will allow us to interpret the impact of the combined action of the transgene and the vector insertion site on cell biology, a factor that until now, because of lack of accessibility, has not been studied in detail.

Individual institutions may provide significant contributions, but a combined international effort will probably be needed to accumulate the amount of data required. Revisions of the existing regulatory guidelines may be helpful only if they promote reasonable standards of comparison and agreements on basic experimental approaches in preclinical research. We propose to consider the collaborative development of preclinical proto-oncogenic worst case models as one basis for dose finding. Thus, developing tools for the best feasible genetic treatment will profit from a dialectic approach that anticipates adverse events by active, hypothesis-driven investigation. While doing so, we need to continue with many of the current clinical trials on the basis of the risk evaluation at hand. It should not be forgotten that for a number of patients, after carefully weighing risks and benefits, the worst case scenario of gene therapy may be to not receive it.

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## Erratum

In the article by Lin et al entitled "The death-promoting activity of p53 can be inhibited by distinct signaling pathways," which appeared In the December 1, 2002, issue of *Blood* (Volume 100:3990-4000), Figure 4C's image lacked 2 unfilled histograms. The correct image for panel 4C appears below.

C

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Current Opinion in Hematology



## Insertional mutagenesis in gene therapy and stem cell biology.

### Hematopoiesis

Current Opinion in Hematology. 14(4):337-342, July 2007.
*Baum, Christopher a,b*

**Abstract:**
Purpose of review: Recent preclinical and clinical studies revealed that the semirandom insertion of transgenes into chromosomal DNA of hematopoietic cells may induce clonal competition, which potentially may even trigger leukemia or sarcoma. Insertional mutagenesis caused by gene vectors has thus led to major uncertainty among those developing advanced hematopoietic cell therapies. This review summarizes novel studies of underlying mechanisms; these studies have demonstrated the possibility of improved gene vector biosafety and generated new insights into stem cell biology.

Recent findings: The characteristic insertion pattern of various retroviral gene vector systems may be explained by properties of the viral integrase and associated cellular cofactors. Cell culture assays and animal models, including disease-specific and cancer-prone mouse models, are emerging that reveal the contributions of vector features and systemic factors to induction of clonal imbalance. Databases summarizing vector insertion sites in dominant hematopoietic clones are evolving as new tools to identify genes that regulate clonal homeostasis.

Summary: Mechanistic studies of insertional mutagenesis by random gene vector insertion will lead to improved tools for advanced hematopoietic cell therapy. Simultaneously, fascinating insights into gene networks that regulate cell fitness will be generated, with important consequences for the fields of hematology, oncology and regenerative medicine.